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SUPPLEMENTARY INFORMATION

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A protein-targeting strategy used to develop a selective inhibitor of the E17K point mutation in the PH Domain of Akt1

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Contents

Supplemental Information

Standard Materials

All amino acids were purchased from Aapptec as the FMOC carboxylic acid with the standard TFA side-chain protecting groups. HATU (2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and PEG_5 (Fmoc-NH-PEG_5-CH_2CH_2COOH, Fmoc-18-amino-4,7,10,13,16-pentaoxaoctadecanoic acid) were purchased from ChemPep. DIEA (diisoproylethylamine), triethylsilane (TES) and TFA (Trifluoroacetic acid) were purchased from Sigma. TentaGel beads were purchased as 90µm S-NH2 beads, 0.29mmol/g, $2.86x10^6$ beads/g from Rapp Polymere (Germany) and Rink Amide resin was purchased from Anaspec.

Peptide Library Construction

Peptide libraries were synthesized on a Titan 357 split-and-mix automated peptide synthesizer (Aapptec) via standard FMOC SPPS coupling chemistry[1] using 90µm TentaGel S-NH_2 beads. Libraries contain 18 D-stereoisomers of the natural amino acids, minus Cysteine and Methionine, at each of five randomized positions and an azide or alkyne *in situ* click handle. At least a five-fold excess of beads is used when synthesizing libraries to ensure oversampling of each sequence. Amino acid side-chains are protected by TFA labile protecting groups that are removed all at once following library synthesis.

Bulk Peptide Synthesis

Bulk synthesis of peptide sequences was performed using standard FMOC SPPS peptide chemistry on either the Titan 357 automated peptide synthesizer (AAPPTEC) or a Liberty 1 microwave peptide synthesizer (CEM Corporation). The typical scale was 300mg on Rink Amide Resin, unless otherwise noted. Peptides were cleaved from the beads with deprotected side-chains using a 95:5:5 ratio of TFA: H_2O: TES. The peptides were purified on a prep-scale Dionex U3000 HPLC with a reverse-phase C18 column (Phenomenex). All peptides are checked for correct mass and impurities using MALDI-TOF MS and are lyophilized to a powder for long-term storage at room temperature. Concentrated peptide stocks for assays are made by dissolving powder in small amounts of DMSO and measuring the A280 absorbance via nanodrop to determine the stock concentration.

Akt1 Wildtype and E17K Mutant Pleckstrin Homology Domain Expression

Akt1 Pleckstrin Homology Domain DNA was purchased from DNA2.0 with codons optimized for expression in *E.coli*. The first 124 N-terminal amino acids from full length Akt1 were used as the PH Domain DNA, and a 6-his tag separated by a thrombin cleavage site were added at the C-terminus of the

protein for purification. In order to make the E17K mutant of the PH Domain, the glutamic acid in position 17 was mutated to a lysine via QuikChange (Stratagene) following all of the manufacturer's protocols. The DNA was synthesized in a pJexpress 414 vector containing an ampicillin resistant gene to be expressed in *E. coli* cells. Protein expression was performed by the Protein Expression Center at Caltech using their standard bacterial expression protocol and purified via Ni-NTA column. The proteins expressed in this manner were used for the immunoprecipitation assays confirming the anchor binding via immunoprecipitation assays and for the biligand screens. These PH Domain proteins were unsuitable for long-term storage under a large variety of tested conditions, so a GST tag was added to hopefully improve the long term stability.

For that reason, the DNA from DNA 2.0 was amplified out of the pJExpress vector via Polymerase Chain Reaction (PCR) to insert the restriction enzyme sites EcoRI and NotI for insertion into a pGEX-4T-1 vector containing a GST tag. The primers used were:

5' - AGAGAATCCATGTCCGACGTCGCGATCGTAAAGGAAGGG – 3'

5' - TCTGCGGCCGCTTAGTGGTGATGATG – 3'

Both the wildtype and E17K mutant DNA were amplified out of the pJExpress vector, restriction enzyme digested, and ligated overnight into a pGEX-4T-1 vector which attached an N-terminal GST tag to the PH Domain protein. BL21-DE3-pLys cells were transformed with the DNA, and the sequences were confirmed via sequencing. An overnight starter colony from each of these plates was grown in 5mL LB+Amp overnight. 4mL of this starter culture was used to inoculate 500mL of LB+Amp and grown to mid-log phase. The cultures were inoculated with 1mM IPTG and grown 5 hours at 28°C. The cells were centrifuged for 10 minutes at 8,000RPM and lysed with lysis buffer (1x TBS, 1mM DTT, 1mg/mL Lysozyme, 1% Triton-X), and left for 30 minutes on ice before flash freezing in liquid nitrogen. Upon thawing on ice, the lysate was sonicated for 5 minutes, then centrifuged for 30 minutes at 10,000 RPM to remove cellular debris. The supernatant was then purified on a HisPur Co column (Pierce) using the manufacturer's recommended protocol. These GST-tagged proteins were used to confirm the biligand binding via immunoprecipitation assays and for the triligand screens. They were also used to obtain the full ELISA curves of all three ligands. These proteins, however, were also not suitable for long term storage and needed to be re-expressed for all assays.

The imaging experiments required that the PH Domain protein be expressed in mammalian cells and have a GFP tag for visualization. Because of this, Akt1 DNA with codons optimized for use in mammalian cells was obtained from InvivoGen as a pUNO-hAKT1 plasmid. The DNA was mutated via QuikChange so both a wildtype and E17K version were on hand. The primers used to clone the DNA from this vector into a TOPO C-terminal GFP mammalian vector were:

5' – AAGATGGGGATGAGCGACGTGGCT – 3'

5' – TCCCCGACCGGAAGTCCATCTCCTC – 3'.

Cloning was done as per the TOPO vector manual. Because the GST-PHD proteins expressed in *E. coli* were still not stable for long term storage, this DNA was used to express the PHD in mammalian cells. The expressions were done by transfecting a suspension culture of HEK-293-6E cells with XtremeGene HD by the Protein Expression Center at Caltech following their standard protocols. These proteins were not purified, and were used as-is out of cell lysates. This protein was used in triligand immunoprecipitation and inhibition assays, and was still not stable for long term storage.

CD Spectroscopy of 33-mer Peptide Epitope

Lyophilized powder of the 33-mer biotin-tagged target fragment that was used for screening was dissolved in 500µL of 1x PBS to a concentration of 0.5mg/mL. Concentrations were estimated by weight and confirmed by A280 measurement using NanoDrop. Experiments were performed using an Aviv 62 CD Spectrometer. The machine was purged for 20 minutes with N_2, then the 1xPBS blank in a 500µL 1cm cuvette was inserted and the machine was purged with N_2 for another 5 minutes. The spectra was acquired by 3 measurements/minute from wavelengths 199-250nm. The 33mer fragment sample was then inserted, purged for 5 minutes, and was measured exactly as the blank. The 33mer cuvette was then removed, and 500µL of 7.0M pH = 2.0 Guanadine-HCl was added to denature the sample. This spectra was acquired as above.

To work up the data, the signal in ΔA from the sample was subtracted from the blank at each wavelength. Then the mean residue molar circular dichroism $\Delta\varepsilon_{MR}$ was calculated from this readout using the number of residues in the fragment (33) and the concentration in mg/mL (0.5 for the folded sample, 0.25 for the denatured sample since it was diluted with Guanadine-HCl) using the equation: **$\Delta\varepsilon_{MR}$= ΔA/((residue # x concentration mg/mL) x l)^2.** The spectra were graphed by plotting this number against the wavelength. As can be seen in **Supplemental Figure S1**, the CD signature for the 33mer peptide resembles that of a β–sheet, which was the expected structure based on the full protein crystal structure. This binding disappeared upon the addition of the denaturing buffer, demonstrating that this is a real structure in the 33mer fragment.

Screen for Initial Anchor Peptide (Supplemental Figure S2)

Screens were performed using a library with 100% Met coupled at the C-terminus for potential MALDI TOF/TOF sequencing[3]. The peptide library was a comprehensive 5-mer containing 18 unnatural D-amino acids, excluding Met and Cys due to stability reasons. The N-terminus contained an azide click handle with varying carbon chain lengths – 2 carbon, 4 carbon and 8 carbon – for in situ click with the Pra on the target 33-mer-epitope fragment. Screens were performed using 300mg of dried library beads swelled at least six hours in 1x TBS (25mM Tris-Cl, 150mM NaCl, 10mM $MgCl_2$, pH = 7.5) buffer.

*Preclear (**Supplemental** Figure S**2A**):*

Swelled library beads were blocked overnight in 5% w/v dried non-fat milk in 1x TBS, then washed with 1x TBS three times. Five milliliters of a 1:10,000 dilution of streptavidin-alkaline

phosphatase conjugate in 0.5% milk in TBS was added to the beads and incubated shaking at room temperature for one hour. The beads were washed with a high-salt TBS buffer (1x TBS with 750mM NaCl) three times, then let shake in high salt buffer for one hour. The beads were then washed three times with BCIP buffer (100mM Tris-Cl, 150mM NaCl, 1mM $MgCl_2$, pH = 9.0) and developed by adding 15mL BCIP buffer plus 13µL BCIP and 26µL NBT (two part system, Promega) After one hour, the purple beads were removed by pipette and discarded. The remaining beads were incubated in NMP 4 hours to remove trace purple precipitate from the BCIP/NBT reaction, then were washed five times with methanol, five times with water, five times with TBS and reblocked overnight in 5% milk.

*Product Screen (**Supplemental** Figure S**2B**):*

Beads remaining from the preclear were washed three times with 1x TBS, then incubated with 5mL of a 100nM dilution of the 33-mer target in 0.5% milk for either 5 hours or 12 hours to allow for an *in situ* click reaction to occur. The beads were then washed three times with 1x TBS and incubated for one hour with a 7M Guanadine-HCl buffer (pH = 2.0) to remove all 33-mer target epitope not attached covalently to the beads. These beads were then washed ten times with 1x TBS, reblocked for two hours in 5% milk, then incubated for one hour with a 1:10,000 dilution of streptavidin- alkaline phosphatase conjugate in 0.5% milk in TBS to detect for the presence of the 33-mer target epitope clicked to a bead. The beads were washed three times with a high-salt TBS buffer, then let shake in high salt buffer for one hour. Afterwards, the beads were again washed three times in BCIP buffer and developed as per the preclear. Purple beads are removed from the screen via pipette as hit beads. These hits were incubated in the guanidine-HCl buffer to remove attached streptavidin, washed ten times with water and sequenced via edman degradation on a Procise CLC system from Applied Biosystems. See **Table S1** for sequences from 5 hour screen, **Table S2** for sequences from 16 hour screen.

All hit ratios were determined by dividing the number of hit beads from the number of beads present in the screen as determined by the mass of the screened library. Hit sequences that could not be accurately called via Edman degradation were still included in this calculation, but were not scaled up and tested as potential candidates.

Sequence Analysis

Hit sequences were analyzed via a peptide analysis algorithm that organized hits based on their hydrophobicity and sequence homology using principal component analysis. The algorithm analyzes a series of peptides and graphs them on a 2D sequence map. Clusters of hits were circled (**Supplemental** Figure S3), and one peptide from each cluster was scaled-up and tested for binding to both wildtype and mutant PH domain. The ligands chosen for scale up were: dqntr, ypwve, eefef, yleaf, and elnhy. Any ligand candidates that were difficult to call on the sequencing were not chosen for scale-up and testing.

Streptavidin – Agarose Immunoprecipitation Assays for Binding Affinity

Immunoprecipitation assays were done on Streptavidin Agarose resin from Invitrogen. The resin was incubated in SpinX tubes (Sigma) for one hour with an excess of N-terminal biotinylated anchor peptide candidates identified via principle component analysis. The anchor candidate coated beads were then blocked for one hour with 5% BSA in TBS, then incubated with either the wildtype or mutant protein overnight at 4C. The protein solutions were spun out, and the resins were washed 3x with 1xTBS + 0.1% Tween-20, 3x high-salt TBS, and 1x TBS before being spun dry and bound proteins were eluted by boiling for 10 minutes in 50uL SDS-PAGE gel loading buffer. The eluted proteins were separated by SDS-PAGE gel, and western blotted for the presence of PH Domain protein.

Relative protein band sizes were analyzed to compare binding between the anchor candidates and were used to determine selectivity for either wildtype or mutant PH Domain (**Supplemental Figure S4**). For selective binding to the E17K mutant protein, yleaf was chosen, as it showed the greatest binding to the mutant with the least binding to the wildtype.

Point ELISAs with Anchor Ligand and 33-mer Epitope – Epitope Targeting Verification

The 33-mer epitope used in screening was resynthesized without the alkyne click handle and with a 6-His tag as an orthogonal tag to the biotin on the anchor ligand. This tag was added after a PEG_5 on the N-terminus of the peptide, and was made and purified as was previously described (**Supplemental Figure S24 and Supplemental** Figure S25).

For these assays, 100nM Biotin-PEG_5-yleaf (**Supplemental** Figure S26) was immobilized for one hour on a Neutravidin-coated ELISA plate (Pierce). The plate was blocked in 5% BSA in 1xTBS overnight at room temperature. The immobilized anchor was then incubated with either 1µM or 100nM wildtype 33-mer epitope or 1µM or 100nM E17K mutant 33-mer epitope for one hour. The plate was washed three times with 1xTBS + 0.1% Tween-20 and tapped dry. The epitope was then detected by a 1:1,000 dilution of an anti-his mouse mAb (ab18184, Abcam) for one hour, washed as above, and then detected with 1:10,000 dilution of an anti-mouse HRP-conjugated goat pAb (Abcam) for one hour. The plate was once again washed and developed with a 1:1 TMB substrate (KMB) for 15 minutes. To graph the data, the blank (epitope and antibodies binding to plate with no anchor ligand present) was subtracted from the triplicate sample values. The fraction bound was found by setting the highest value to 100% and normalizing the rest accordingly. The triplicate values were then graphed (**Fig. 2c**) with their error bars and the p-values were calculated by GraphPad.

HPLC-detected Immunoprecipitation Assays – Epitope Targeting Verification

Immunoprecipitation assays with the biotinylated anchor and his-tagged 33-mer epitope were performed to verify epitope targeting. In these assays, biotinylated anchor ligand was used to pull the WT or E17K mutant fragments from solution. As with the full-protein assays, the biotinylated anchor

ligand was incubated for one hour with 50µL of streptavidin agarose slurry that had been washed three times with 1xTBS. The anchor ligand was washed out, and the resin was blocked for an hour in 5% BSA in 1xTBS. 200µL of a 50µM solution of the His-tagged 33-mer epitope in 1xTBS was added to the blocked resin and this was incubated overnight (~16 hours) at 4°C. Because small peptide fragments like the 33-mer epitope are difficult to transfer to and detect on the nitrocellulose membrane as for a traditional Western blot, the amount of binding in these assays was detected via HPLC. In order to do this, the bound 33-mer peptide fragments were washed three times with 1xTBS + 0.5% BSA and one time with 1xTBS. The resin was then incubated with 200µL of the 7M pH = 2.0 Guanadine-HCl buffer used to strip beads in the screen. The Guanidine buffer was spun out of the beads in Spin-X tubes and injected onto a Beckman Coulter semi-prep HPLC with an analytical scale reverse phase C18 analytical column. The peak seen on the HPLC (**Supplemental Figure S5**) illustrated how much of the 33-mer epitope bound to either the yleaf anchor or to blank beads.

These assays in conjunction with the point ELISAs described above demonstrate the binding of the anchor ligand to the epitope that was used for screening in a variety of different conditions. The results conclusively demonstrate that the yleaf anchor not only binds to the epitope fragment, but is selective for the single amino acid E17K mutation on both the full protein and fragment.

Ligand-Directed Tosylate Labeling Experiments

For these assays, the yleaf anchor on a C-terminal CPP was appended with an N-terminal FMOC-piperidine-4-carboxylic acid as a linker on 300mg of rink amide resin in NMP using standard FMOC amino acid coupling techniques. The resin was equilibrated in anhydrous DCM and 250µL of 3-(chlorosulfonyl)benzylchloride was added with 450µL of DIEA and shook for 30 minutes at room temperature. Then 250µL of 2-(2-(2-aminoethoxy)ethoxy)ethanol, 450µL of DIEA and 19mg DMAP in anhydrous DMC were added and shook overnight. The resin was washed and equilibrated in NMP and 2eq Cy5 carboxylic acid (Lumiprobe) was coupled at 37°C overnight using standard FMOC coupling techniques. The resin was washed, TFA cleaved and HPLC purified as usual.

In order to label the protein, 50µL of full-length GST-E17K Akt1 from SignalChem was treated with 10x molar excess of the anchor ligand with the tosylate dye label and incubated for two days at room temperature. The 10x excess required was determined through a separate experiment in which 1x, 4x, and 10x quantities of labeling arm with biotin label were incubated with the protein and blotted for the presence of biotin. The results of this gel and labeling experiment can be seen in **Supplemental Figure S7.** The mixture was lyophilized after two days and then denatured by boiling in SDS-PAGE loading buffer. The labeled protein was run alongside an unlabeled control on an Any-KD gel from Biorad, then imaged on an Odyssey fluorescent gel reader at 700nm emission (**Fig. 3b**). After confirming that labeling had occurred, the gel was stained with BioSafe Coomassie blue stain (BioRad) and the blue protein bands were cut out. The gel pieces were trypsin digested using the Pierce In-gel Digest Kit. The tryptic fragments from both the unlabeled and labeled protein digests were lyophilized to concentrate them, taken up in 2µL of 50% H_2O/50% acetonitrile and were analyzed by MALDI-TOF MS (**Supplemental** Figure S8 **and** Figure S9).

Initially, analyses were performed by taking any peak that was present in the labeled protein sample that was not present in the unlabeled sample. The weight of the dye labeling arm – 552.37 g/mol – was subtracted from these peaks and the corresponding tryptic fragment was located. This provided four potential fragment candidates that were all located near the 33-mer epitope in the PH domain of the protein. Next, every MALDI peak in the labeled sample was analyzed by subtracting the weight of the dye label and comparing it to a potential tryptic fragment (**Supplemental Table S3**). One other fragment was identified using this method, and corresponded to the doubly labeled peak of one of the previously identified labeled fragments. These results confirmed multiple previous experiments done using LC/MS techniques that proved not strong enough to fragment the tryptic peptides into individual amino acids.

These tryptic peptide samples were then analyzed by MALDI-TOF/TOF MS to identify the exact amino acid that contained the dye label. Only YFLLK was able to be successfully fragmented (**Supplemental** Figure S10), and the TOF/TOF confirmed that the tyrosine was the label-containing amino acid. This confirms the results seen in the original publication[4] that only Y, H, and E nucleophilic amino acids are labeled using this technique. The remaining tryptic fragments all contain at least one of these amino acids, with the double labeled fragment containing two.

The labeling sites were then plotted onto a Pymol image (**Fig. 3c**) that combined the Akt1 protein (PDB: 3096) and the E17K PH Domain (PDB: 2UZR) with the N-terminal GST tag (PDB: 1UA5) that was present on the full-length protein from SignalChem that was used in these labeling assays. This Pymol-made fusion protein was used to approximate what the commercial protein looked like in solution and give an idea of the extent of the selectivity of this assay. The concentration of labeling sites only surrounding the epitope demonstrate the exclusive binding of this ligand in solution.

Details on Tryptic Fragment Workup for Labeling Experiment

All of the peaks from the MALDI-TOF spectra of the labeled tryptic digests were analyzed for their potential to contain a dye label. The MALDI spectra was manually calibrated to ensure the least possible error. Each peak was then analyzed by zooming in on the spectra on the computer and obtaining the exact mass for the monoisotopic peak, which is recorded as “MALDI peak” in

***Table* S3** below. The mass of the dye, 552.37g/mol, was subtracted from this peak, and it was compared to the closest possible theoretical tryptic digest fragment ("Digest"). The "expected" mass of the digest plus the dye was calculated and subtracted from the observed mass, "MALDI peak", and the absolute value of this difference was recorded in "P/M 1". The peak area was obtained from the MALDI data and added to the spreadsheet as "Peak Area" to allow for a cutoff (4500) of any peaks that looked to be within the noise. Any peak below this value is shown in red italics, and was not considered for this study. Any peak that was within 0.1% of the mass of the expected digest mass was considered to be within error of the instrument and was considered a hit dye-labeled fragment. There were no new peaks seen using this method than were discovered by looking for peaks that grew in from the unlabeled MALDI to the labeled MALDI. The labeled sites seen in this MALDI-TOF experiment were all seen previously in at least 2 LC/ESI-MS experiments attempting to identify the labeled region.

The peak at ~2212 was not seen on the unlabeled mass spec, but is seen on the labeled fragment and was considered a hit. 2211 is also, however, a common mass seen for trypsin. We do see this particular unlabeled fragment fly in the MALDI-TOF MS (1659), and know from the ESI-MS experiments that this is a site that can be labeled. In attempting to zoom in for the monoisotopic mass, we see a broad peak with no clearly identifiable mass peak – unlike all of the other peaks in the spectrum which showed the distribution of masses very clearly. This lead us to believe that we are, in fact, seeing this peak labeled in the MALDI, especially as this site was seen as labeled by the ESI. The ESI labeling experiments were also done using a labeling arm containing biotin and not Cy5, so this mass did not overlap with trypsin in these experiments. We just cannot exactly call this mass in the MALDI due to the similarity of this peak to that of trypsin.

Images of anchor ligand in HEK-293T cells expressing PH Domains

These experiments were designed to visualize the dye-labeled anchor ligand in cells overlapping with the GFP-labeled PH Domain proteins. For this reason, the yleaf anchor ligand was synthesized with an N-terminal PEG_5, TAT (YGRKKRRQRR), and Cy5 dye (**Fig. 2a**). GFP-tagged protein DNA was also cloned as described above. HEK-293T cells were grown in DMEM media supplemented with 10% FBS (both Invitrogen), 100x non-essential amino acid solution (Sigma), and PenStrep antibiotic (Invitrogen). Once the cells reached ~80% confluency, they were treated with trypsin to remove from the plate and split into small wells with a D-poly-lysine (BD) coverslip at approximately a 50% confluency in 1mL total volume. The cells were allowed to attach to the coverslips for approximately 24 hours, then were transfected to express either wildtype GFP-PH domain or E17K mutant GFP-PH domain proteins using XtremeGene HD transfection agent at a ratio of 3:1 transfection agent to DNA. Several wells were left untreated as no protein blanks (**Supplemental Figure S*14***). The cells were given 24 hours to express protein. They were then serum starved for one hour in DMEM media prepared as above, but without the FBS. After one hour, the Cy5-labeled anchor was added to the wells to a final concentration of 50nM. As the HEK-293T cells are expressing endogenous Akt1 protein, this level was adjusted to give the lowest background signal possible. The protein blank cells were also incubated with 50nM of the yleaf anchor to ensure that binding was due to the presence of the E17K mutant protein (**Supplemental**

Figure S*14*). A blank of PEG5-TAT-Cy5 was also added to wells expressing either wildtype or E17K mutant to ensure that ligand binding was due to the presence of the yleaf anchor. After a one hour incubation with the peptide, the cells were washed once in serum starved media, then incubated one hour in serum starved media to wash out any excess peptide (see **Supplemental Figure S13** for a comparison of peptide with and without the one hour wash step). During this time, the cells were also treated with 10µg of Hoescht 33342 dye to stain the nuclei. The cells were then washed twice with cold PBS buffer, fixed with 10% Neutral Buffered Formalin Solution (Sigma) and glued onto microscope slides. Images were taken on a Zeiss LSM 510 Meta NLO with Coherent Chameleon confocal microscope. A 40x Plan-apochromat lens was used. The laser intensity and gain were fixed for all pairs of images between wildtype and mutant samples to ensure that the differences seen were not artificially created.

The images were analyzed by calculating the Pearson correlation coefficient in order to determine the degree of co-localization of the GFP-tagged proteins and the Cy5 anchor ligand in cells. These calculations were performed by importing the images into ImageJ and splitting the channels. The red and green channels were then analyzed for their Pearson coeeficient using the Just Another Colocalization Plugin (JACoP). Four representative images for each protein were analyzed and averaged to determine the final reported correlation coefficient. The values for these WT images were: 0.213, 0.090, 0.080, and 0.19. The values for the E17K images were: 0.448, 0.305, 0.674, and 0.434. The final values were determined to be $r = 0.143 \pm 0.059$ for the WT and $r = 0.464 \pm 0.133$ for the E17K mutant, and are statistically significant ($p = 0.0045$).

The unwashed images in **Supplemental Figure S13** were made using HEK-293T cells grown on coverslips expressing WT and E17K protein as described previously. The cells were serum starved for one hour, then incubated with 500nM ligand for one hour. The cells were then washed twice in cold TBS buffer and fixed as described previously.

Screen for Biligand Peptide (Supplemental Figure S16)

The anchor ligand determined above – yleaf – was scaled up with a biotin on the N-terminus for detection, a PEG_5 linker between the biotin and the peptide, and a d-propargylglycine (Pra) on the C-terminus as the *in situ* click handle (Biotin-PEG_5-yleaf-Pra, **Supplemental Figure S26**). Screens were done using a library with 100% Met coupled at the C-terminus for potential MALDI TOF/TOF sequencing. The library consisted of a comprehensive 5-mer containing 18 unnatural D-amino acids, excluding Met and Cys due to stability reasons. The N-terminus contained an azide click handle with a 4 carbon chain ($Lys(N_3)$)– for in situ click with the Pra on the anchor peptide. Screens used 300mg of dried library beads swelled at least six hours in 1x TBS (25mM Tris-Cl, 150mM NaCl, 10mM $MgCl_2$, pH = 7.5) buffer.

*Preclear (**Supplemental Figure S16A**):*

Swelled library beads were blocked overnight in 5% w/v dried non-fat milk in 1x TBS, then washed with 1x TBS three times. The beads were incubated with a 7.15µM solution of the anchor

peptide – Biot – PEG_5 – yleaf – Pra for one hour then washed three times with 1xTBS. Five milliliters of a 1:10,000 dilution of streptavidin-alkaline phosphatase conjugate in 0.5% milk in TBS was added to the beads and incubated with shaking at room temperature for one hour. The beads were washed with a high-salt TBS buffer (1x TBS with 750mM NaCl) three times, then let shake in high salt buffer for one hour. The beads were then washed three times with BCIP buffer (100mM Tris-Cl, 150mM NaCl, 1mM $MgCl_2$, pH = 9.0) and developed by adding 15mL BCIP buffer plus 13µL BCIP and 26µL NBT. After one hour, the purple beads were removed by pipette and discarded. The remaining beads were incubated in NMP four hours to remove trace purple precipitate from the BCIP/NBT reaction, then were washed five times with methanol, five times with water, five times with TBS.

*Target Screen (**Supplemental Figure S16B**):*

The clear beads remaining from the preclear were blocked in 5% milk in 1x TBS for two hours. They were then washed three times with 1x TBS. A pre-incubated solution of E17K mutant protein (715nM) and anchor ligand (7.15µM) in 3mL of 0.5% milk was added to the blocked library beads and incubated for either 5 hours or overnight to allow an *in situ* click reaction to occur. In the morning, the beads were washed three times with 1x TBS, then incubated with a 1:4,000 dilution of an anti-His alkaline phosphatase conjugated antibody (Abcam) in 0.5% milk for one hour. The beads were then washed three times with a high salt TBS, then incubated on the shaking arm for one hour with the high salt buffer. They were then washed three times with BCIP buffer and developed as previously. Hit beads turned purple and were removed and washed in NMP for four hours to decolorize, then guanidine-HCl to denature and remove and remaining protein. The beads were then washed ten times with water and blocked in 5% milk overnight.

*Off-Target Anti-Screen (**Supplemental Figure S16C**):*

The beads from the target screen were washed three times with 1x TBS, then incubated with the off-target, wildtype PH Domain protein in 0.5% milk for one hour on the shaking arm at room temperature. The beads were washed three times with 1x TBS, then incubated with a 1:4,000 dilution of Anti-His alkaline phosphatase conjugated antibody in 0.5% milk for one hour at room temperature. They were then washed three times with high salt buffer and let shake for one hour in high salt at room temperature before being washed three times with BCIP buffer and developed as previously. The beads that turned purple bind to both mutant and wildtype protein or to the anti-his antibody and were set aside. The beads that remained clear were picked and washed with guanidine-HCl to remove any bound proteins and blocked in 5% milk overnight.

*Product Screen (**Supplemental Figure S16D**):*

The beads specific for the mutant PH domain were washed three times with 1x TBS. They were then incubated with a 1:10,000 dilution of streptavidin – alkaline phosphatase conjugate in 0.5% milk for one hour. The beads were washed three times with high salt TBS then let shake for one hour with high salt buffer before being washed three times with BCIP buffer and developed as previously. The beads

that turned purple contained the anchor peptide covalently bound to the bead and had formed a protein-catalyzed *in situ* click reaction. These beads were collected and stripped with guanidine-HCl for one hour, washed ten times with water, and sequenced via Edman degradation as per the anchor candidate hits. There were 22 total hit beads (***Table S4***). Upon sequencing, the selected hits ended up containing only four amino acids instead of five. One of the random amino acids must have not coupled upon library synthesis, but the sequences were used anyways.

Streptavidin-Agarose Immunoprecipitation Assays to Test Biligand Candidates

Four biligand candidates were chosen based on their hydrophobicity and sequence homology using principal component analysis (**Supplemental Figure S17**). Biligands were synthesized by coupling the 2° ligand onto Rink Amide Resin on the Titan peptide synthesizer. The amide group on the end of the Lys(N_3) was capped by shaking the resin with 2mL acetic anhydride, 2mL NMP and 0.5mL DIEA for three times 10 minutes each, then washed with NMP. FMOC-Propargylglycine-Otbu (Pra) was clicked onto the Lys(N_3) on the 2° ligand by incubating 2 equivalents of the Pra amino acid with 2 equivalents CuI and 2 equivalents ascorbic acid with 1 equivalent azide on resin in 20% piperidine/NMP for 3 hours. The resin was washed five times 4mL with a chelating solution of 1g sodium diethyldithiocarbamate in 20mL NMP and 1mL DIEA. The anchor was then built onto the 2° ligand on bead, and an N-terminal PEG_5-biotin tag was added. Assays were performed exactly as for the anchor ligands, except for two key differences. The biligand assays were done with 6ug of GST-tagged PHD protein, instead of the untagged PHD that was used in the anchor immunoprecipitations. The immunoprecipitation assays were also conducted out of 1% serum in 1x TBS, as opposed to just 1x TBS, and results can be seen in **Supplemental Figure S18**. As can be seen from the western blot, all of the biligands improved upon the anchor binding to the E17K PHD protein, but yleaf-yksy showed the highest signal for the E17K protein while still demonstrating the lowest off-target signal to the WT protein.

Screen for Triligand Peptide (Supplemental Figure S19)

The best biligand candidate as determined in immunoproecipitation assays – yleaf-Tz-yksy - was scaled up with a C-terminal PEG_5-biotin for detection during the assay by coupling PEG_5 onto NovaTag Biotin resin (EMD). Then Lys(N_3)-yksy was coupled onto the resin on the Titan peptide synthesizer, and FMOC-Pra-Otbu was clicked on as above. The resin was then placed back on the Titan to synthesize the remaining "Lys(N_3)-yleaf" portion – the Lys(N_3) serving as the click handle for the triligand screen. The biligand was then TFA cleaved from the resin and purified (**Supplemental Figure S31**). The screens were completed using a random 5 D-amino acid library with a C-terminal D-propargylglycine alkyne click handle, and were otherwise performed exactly as for the biligand, including all concentrations. Only 3 hit beads were discovered in this screen, and the first hit had a nonsensical sequence so could not be used. See **Table S5** for hits. Both of the hits were scaled up and tested for binding using ELISA assays (**Supplemental Figure S20**) using the protocol for the full ELISA curves for the ligands.

Full ELISA curves for Ligands

The full curve ELISAs were obtained using streptavidin coated ELISA plates (Pierce). The ligands – anchor, biligand, triligand and "eflya" scrambled anchor peptide blank - were laid down at a concentration of 1µM for one hour. Two lanes of each ligand were used on the plate for both proteins – WT and E17K GST-PH Domain. The plates were blocked with 5% BSA for two hours. Dilutions of both WT and E17K GST-PH Domain proteins were made in 0.5% BSA in 1xTBS starting from 1µM – 0.5nM by serially diluting 1:2 down a series of 8 samples. For each ligand, a no protein blank was also used. The proteins were incubated with the blocked plate for one hour, washed 3x with 1xTBST + 0.5% BSA and tapped dry, then detected with a 1:10,000 dilution of an HRP conjugated anti-GST pAb. The plate was again washed 3x with 1xTBST and tapped dry. It was developed with a 1:1 solution of TMB substrate and development was stopped with 1M H_2SO_4 and read on a plate reader. The curves in **Supplementary** Figure S21 were plotted by normalizing the signal by the blank wells, and were fitted to a Hill function in GraphPad using a common saturation and slope (Bmax = 1.466 +/- 0.03, h = 0.7383 +/- 0.025).

Fluorescence Polarization Assays

The yleaf anchor and triligand were synthesized with Cy3 for fluorescence polarization (FP) assays. The assays between the anchor ligand and the peptide epitopes were done by making a 100 µM starting concentration of each WT and E17K mutant epitopes, and diluting it 4x down a series of 12 wells in a black 96 well polystyrene plate. Cy3-anchor ligand was added to the wells for a final concentration of 200nM, and the plate was incubated for one hour at room temperature with shaking. The plate was read on a Flexstation3 fluorescent plate reader.

The full protein assays were completed by making a 1µM starting protein concentration and diluting 2x down a series of 12 wells in a black 96 well polystyrene plate. Ligand (either Cy3-anchor or Cy3 triligand) was added to the wells for a final concentration of 133nM for the E17K protein and 400nM for the WT protein. The plate was incubated at room temperature for one hour with shaking, and read on a Flexstation3 plate reader.

The data were fitted by subtracting the average of the low concentration baseline to zero in order to use the Hill fit. Using GraphPad Prism, the curves were fitted to a Hill function using a common saturation point.

For all FP assays, the noise level is high when there is little binding – i.e. when the protein concentration is very low, for the assays of the fluorescent yleaf anchor ligand with WT protein. This likely arises from a small amount of PCC Agent adsorbed to the microwell surface. We thus validated the yleaf-WT protein binding assays via three independent measurements (**Supplemental** Figure S6).

The anchor-epitope assay can be seen in **Figure 2d.** The anchor-full protein assay can be seen in **Figure 2c,** and the triligand-full protein assay is **Figure 5c.**

Point ELISA assays for Triligand Binding to Akt1 and Akt2 Wildtype and E17K Mutant

These assays were conducted to test the binding of the triligand to the off-target Akt2 wildtype and mutant proteins. For this assay, all samples were taken in triplicate for statistical purposes. Triligand peptide was first immobilized onto Neutravidin ELISA plates (Pierce) for one hour. A scrambled anchor peptide, eflya, was used as the no-ligand blank, as the GST proteins has significant background binding to a blank Neutravidin plate. The plates were then blocked with 5% BSA overnight. Protein was laid down at a concentration of 100nM for samples wells and the blank, scrambled peptide wells. GST protein alone (Abcam) was also incubated with the triligand and scrambled peptide as a control. The proteins were incubated for one hour, then washed three times with 1xTBST. The protein was then detected with 1:10,000 anti-GST mouse mAb (Fisher, #MA4-004) for one hour, washed three times with 1xTBST and developed with a 1:1 mixture of TMB substrate for ten minutes. The samples were plotted by subtracting the blanks and averaging the sample wells. The highest signal was considered 100% binding, and the other samples were normalized accordingly. The homology of the PH Domain between $Akt1^{E17K}$ and $Akt2^{E17K}$ is 79% as calculated by a pairwise sequence analysis using Blast2Seq between the Akt1 E17K structure (2UZR) and the Akt2 PHD structure (1P6S). This assay can be seen in **Supplementary** Figure S22**.**

Point ELISAs for Anchor and Triligand Binding in Human Serum

These assays were conducted to test the off-target interactions of the anchor and triligand. These point ELISAs were conducted in 1% and 2% human serum to determine if the binding of the protein to its desired target is lessened by the presence of high amounts of other human proteins. For this assay, Anchor and Triligand peptide were first immobilized onto Neutravidin ELISA plates (Pierce) for one hour. A scrambled anchor peptide, eflya, was used as the no-ligand blank, as the GST proteins has significant background binding to a blank Neutravidin plate. The plates were then blocked with 5% BSA overnight. Protein was laid down at a concentration of 150nM for samples wells and the blank, scrambled peptide wells in either 0%, 1% or 2% human serum in TBS buffer. The proteins were incubated for one hour, then washed three times with 1xTBST. The protein was then detected with 1:10,000 anti-GST mouse mAb (Fisher, #MA4-004) for one hour, washed three times with 1xTBST and developed with a 1:1 mixture of TMB substrate for ten minutes. The samples were plotted by subtracting the blanks (elfya scrambled peptide binding to protein). This assay can be seen in **Supplemental Figure S23**.

PIP_3 Agarose Immunoprecipitation Inhibition Assays

PIP_3 Agarose beads (Echelon) were used to detect for the inhibition of PH Domain binding to its substrate, PIP_3, upon incubation with the anchor candidate peptide ligands. To test the inhibition of each of the ligands, anchor, biligand and triligand, 20µL of resin slurry was added to each of four tubes

and washed three times with 1x TBS. Protein, 2µg (234nM) of E17K mutant, was pre-incubated for one hour at room temperature with either DMSO (no protein blank), anchor, biligand or triligand at 2.38µM (10x in relation to protein) in 200µL of 1x TBS. For the control, mutant PH Domain was incubated with 1x TBS and 1µL MSO to mimic the ligand conditions. These protein samples were then added to PIP_3 agarose in a Spin-X tube and incubated at room temperature for two hours. The resin was washed three times with 1x TBS with 0.25% IGEPAL CA-630, spun out to dry completely, then denatured with 50µL 3x SDS gel loading buffer for 10 min at 95°C. The gel loading buffer was spun out of the resin and detected via western blot as per the streptavidin - agarose immunoprecipitation assays. Inhibition was indicated by a decrease in the amount of PH Domain that was pulled from solution by the resin. See **Figure 5d**.

Expanded inhibition blots with either WT of E17K mutant protein were performed in a similar fashion. Twelve tubes of 20µL of PIP3 agarose were washed three times with 1x TBS. 2µg of either wildtype or mutant PH Domain-GFP protein (234nM) in 200uL 1xTBS were pre-incubated for 30 minutes with differing concentrations of triligand: 0.1eq (23.4nM), 1eq (234nM), 10eq (2.34µM), 100eq (23.4µM), and 1000eq (234µM). The protein and triligand solutions were then incubated with the PIP3 resin for 2 hours at room temperature. The resins were washed, eluted, and blotted as per all PH Domain western blots. See **Figure 5e**.

Figures and Tables:

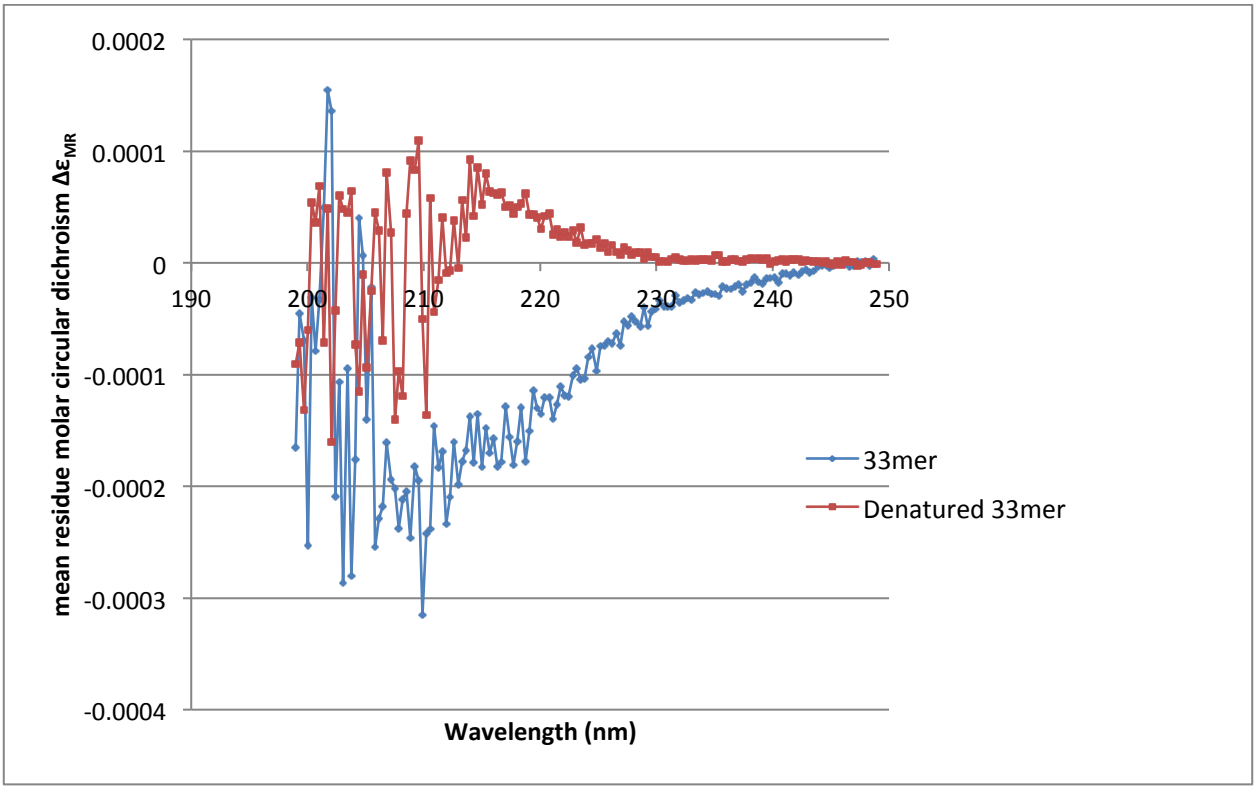


Figure S1: CD Spectra of 33mer fragment, folded and denatured

The blue spectra indicated the 33mer target fragment that was used in screening. The dip at 217nm indicates β-sheet formation, as to be expected from the protein structure. The CD was also collected under denaturing conditions to ensure that the peak at 217nm was due to peptide structure. The messy spectra from 200-210nm are probably due to the biotin tags attached to the fragment.

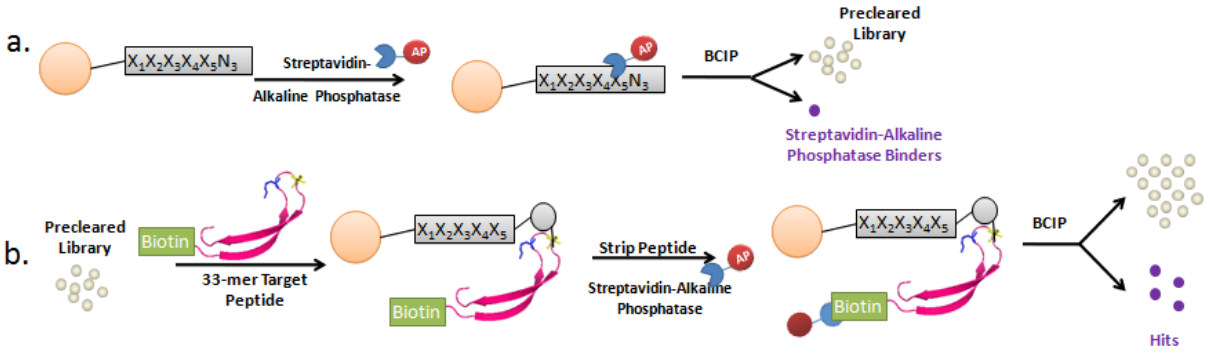


Figure S2: Screening Strategy for Anchor Ligand Determination

(a) Preclear: Library beads are incubated with streptavidin - alkaline phosphatase conjugate to remove any library beads that bind to this or the BCIP reagents. **(b)** Screen: Precleared library beads are incubated with the 33-mer target peptide containing an azide in situ click handle. The fragment catalyzes triazole formation between the alkyne on the 33-mer target and the azide on beads that contain peptide sequences that bind specifically to the 33-mer in a close enough proximity to the alkyne substitution for a click reaction to occur without copper. The unclicked peptide is then stripped from the beads and the remaining covalently attached 33-mer is detected by streptavidin – alkaline phosphatase with BCIP development.

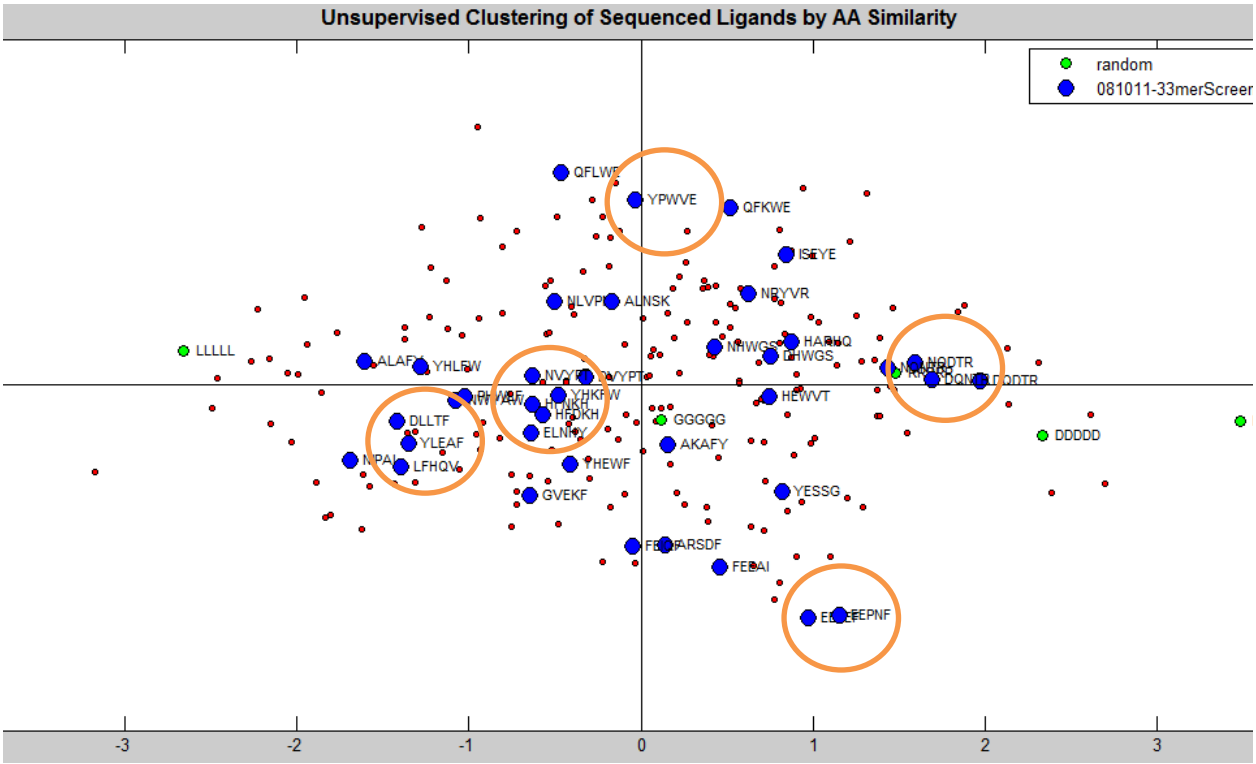
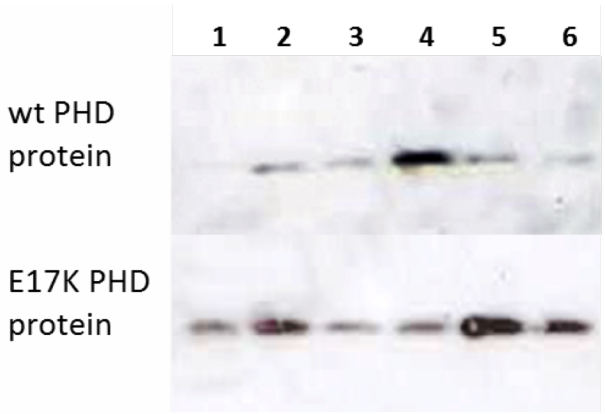


Figure S3: Unsupervised Clustering of Anchor Sequence Ligands by AA Similarity

Hit sequences from the anchor screen were analyzed by their hydrophobicity and sequence homology using principal component analysis. Circled clusters indicate regions where a peptide was selected and scaled-up as a possible anchor sequence. The potential anchor sequences that were tested are: dqntr, ypwve, eefef, yleaf and elnhy.



Lane	description
1	Blank, no ligand
2	Biotin – dqntr
3	Biotin – ypwve
4	Biotin – eefef
5	Biotin – yleaf
6	Biotin - elnhy

Figure S4 Streptavidin-Agarose Immunoprecipitation Assays for Anchor Ligand Binding Affinity Streptavidin-agarose was incubated with a panel of potential anchor sequences that were synthesized with biotin tags. These resins were then incubated with either wildtype or E17K Mutant PHD to measure the amount of immunoprecipitation for each potential anchor ligand.

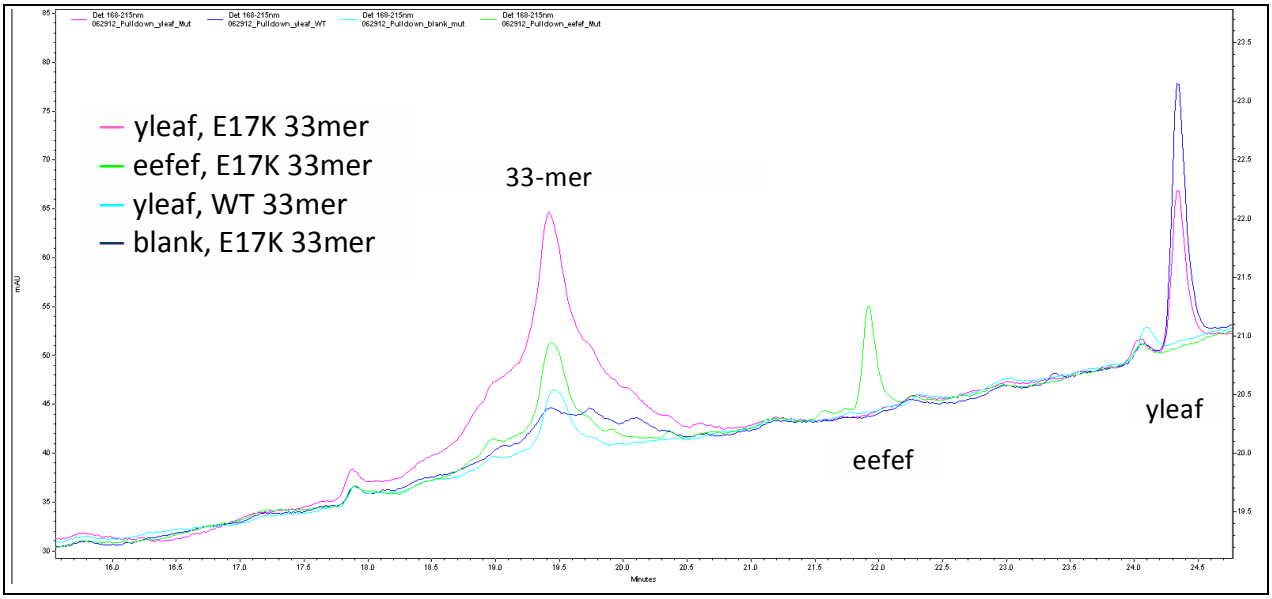


Figure S5: HPLC-detected Immunoprecipitation Assays for Epitope Targeting Verification The major peak indicated by "33-mer" demonstrates the amount of 33-mer fragment that was pulled down by either the "yleaf" anchor ligand or an off-target ligand "eefef", both of which can be seen in the HPLC due to the Guanadine elution stripping them from the resin with the fragment. The amount of 33-mer fragment that each peptide was able to pull out of solution can be seen by the size of the peak on the HPLC trace.

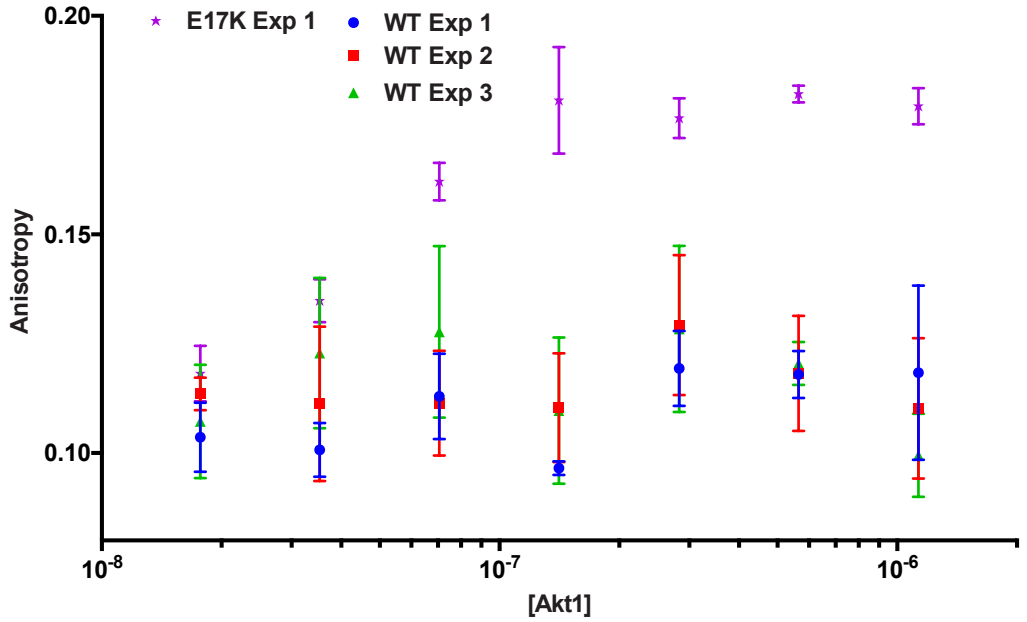


Figure S6: Demonstration of Consistency in Baseline for WT Fluorescence Polarization Anisotropy Raw Data

The WT protein binding to the anchor ligand over three separate fluorescence polarization experiments.

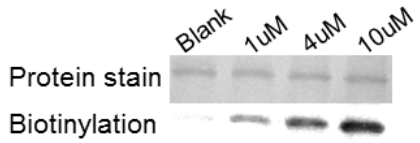


Figure S7: Protein stain and labeling blot

This represents a labeling experiment performed on GST-PHD protein using the anchor ligand with a labeling arm that transfers a biotin onto the target protein. The anchor ligand was incubated with 1uM of protein at the concentrations shown above. The blank includes no labeling arm. The protein stain is a Ponceau stain, and the western blot for biotinylation was performed using an anti-biotin-HRP antibody.

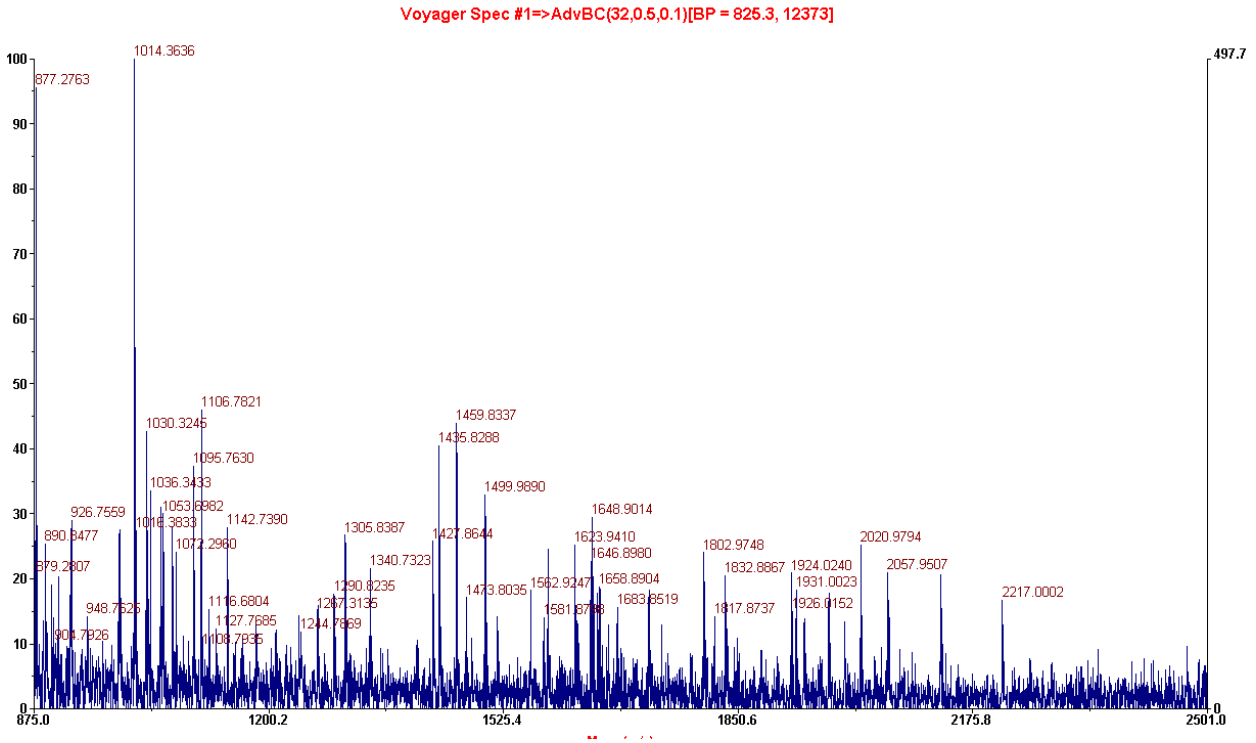


Figure S8: MALDI -TOF of Cy5 unlabeled trypsin digests from tosyl labeling experiments

The unlabeled GST-E17K PHD- Akt1 protein was trypsin digested and the fragments were analyzed by MALDI-TOF MS. See *Table S3* and detailed methods description for the data comparison and workup.

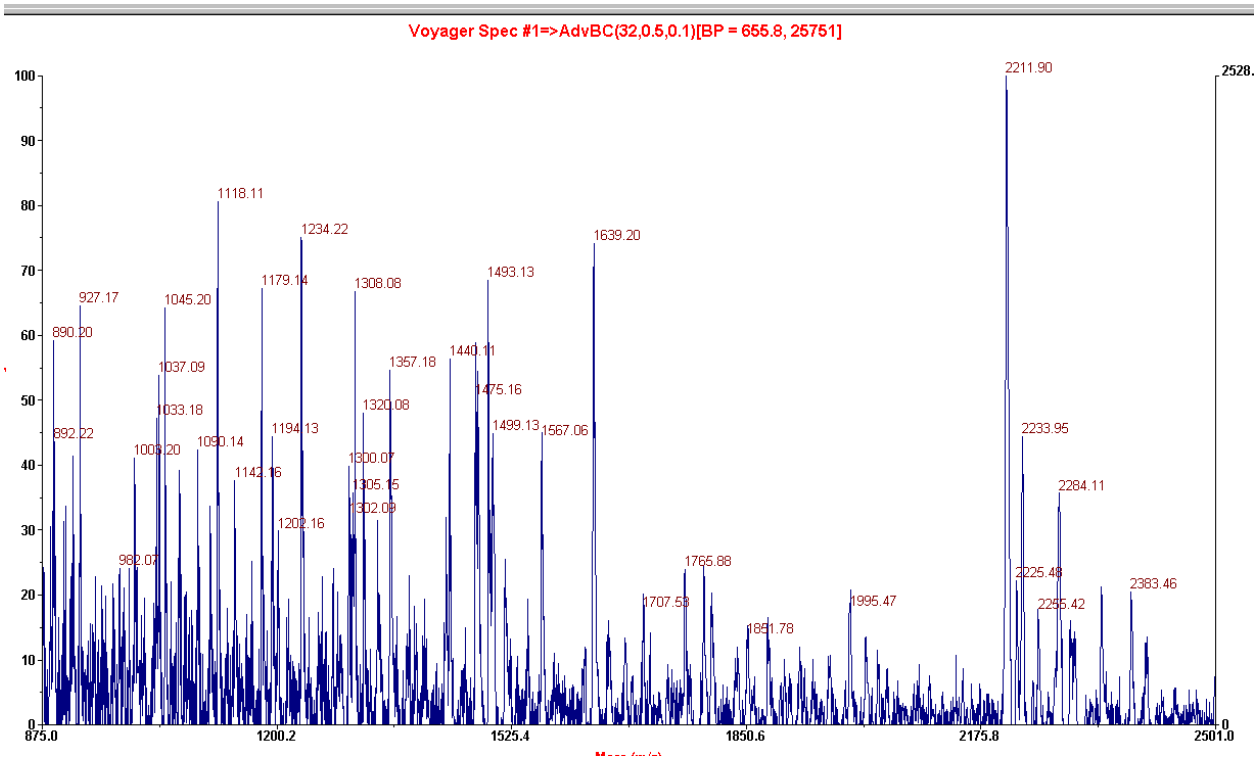


Figure S9: MALDI -TOF of Cy5 dye-labeled trypsin digests from tosyl labeling experiments.
The GST-E17K PHD- Akt1 protein that had been labeled by the anchor – tosyl – Cy5 was trypsin digested and the fragments were analyzed by MALDI-TOF MS. These fragments were compared to those seen in Figure S8 to determine which ones contained the addition of the dye molecule. Those fragments were further analyzed by MALDI-TOF/TOF MS. See **Table S3** and detailed methods description for the data comparison and workup.

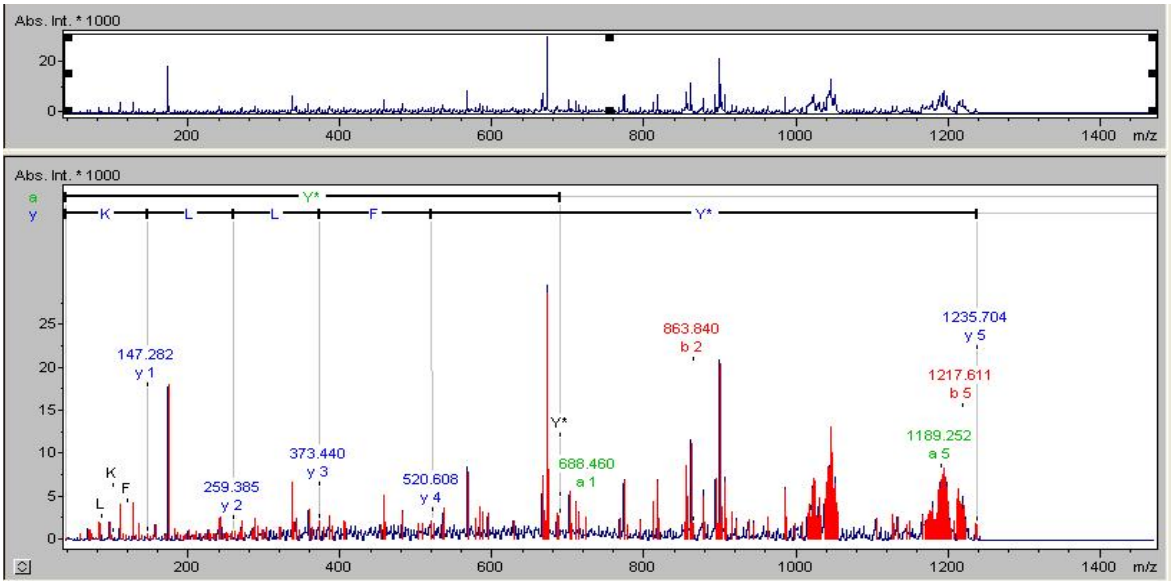


Figure S10: MALDI-TOF/TOF Cy5 dye-labeled YFLLK fragmentation

The YFLLK – Cy5 Labeled trypsin fragment analyzed by MALDI-TOF/TOF MS. The fragments shown above demonstrate that the Cy5-dye is on the Y amino acid, which corresponds to the results found by authors of the original technique[4].

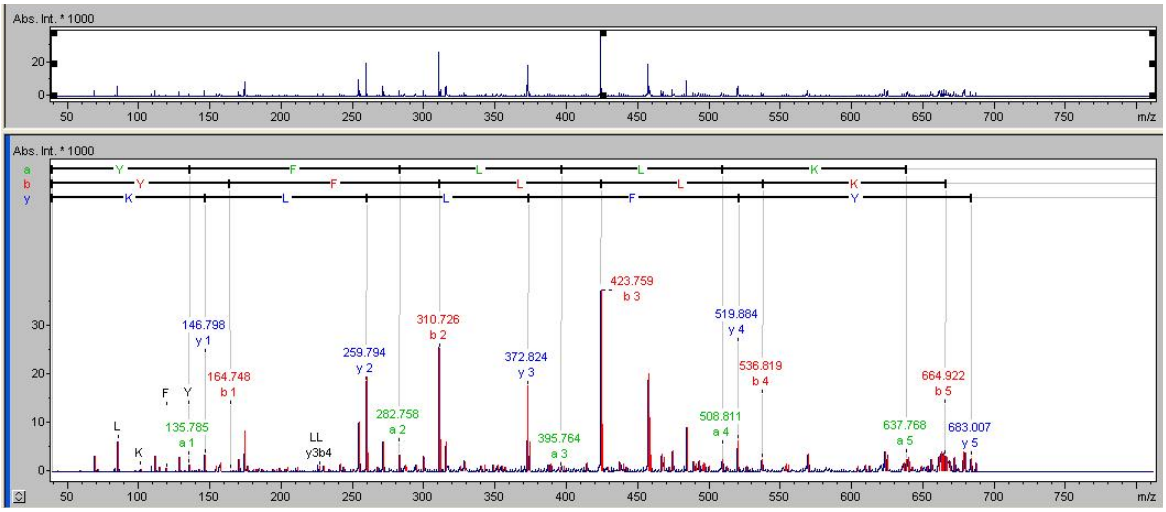


Figure S11: MALDI-TOF/TOF unlabeled YFLLK fragmentation

The unlabeled YFLLK trypsin fragment analyzed by MALDI-TOF/TOF MS. The fragments shown demonstrate that we are able to use MALDI-TOF/TOF to readily fragment the trypsin-digested proteins in order to determine the location of the dye label.

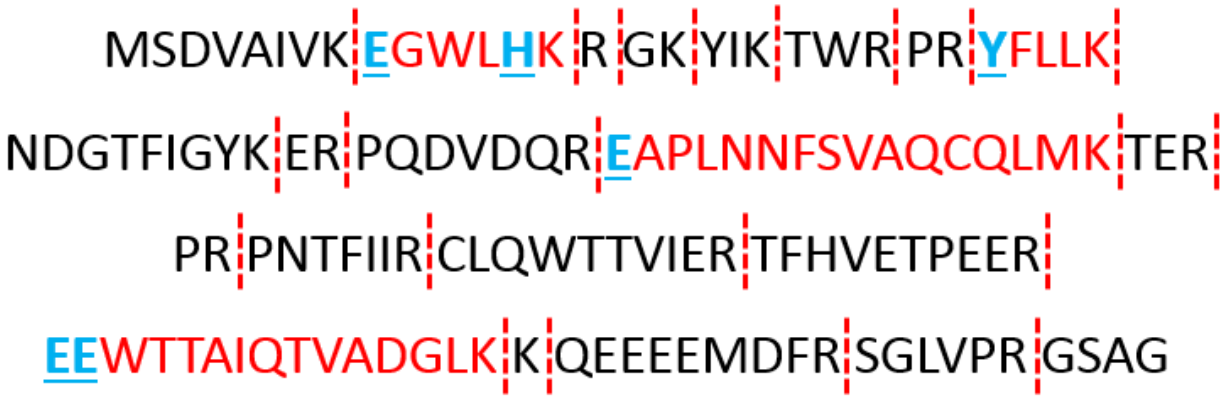
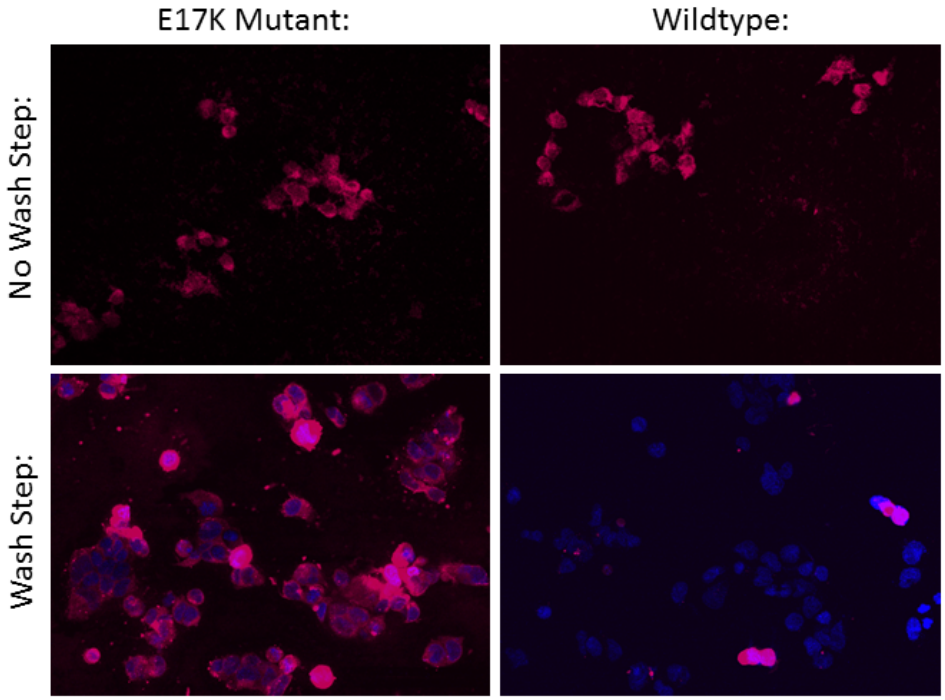


Figure S12: Sequence of PH Domain with Trypsin Digests and Labeled Fragments

The sequence of the PH Domain is digested by trypsin at the dashed lines. The fragments that were seen to contain a label are highlighted in red and the sites that may contain a label are highlighted in cyan. Note that the first fragment has two possible labeling sites, and the peaks for both the singly and doubly labeled MALDI fragments were seen.



	E17K Mut.	Wildtype
No wash avg Cy5 intensity/cell	1515.1 ± 89.7	1302.6 ± 34.6
Wash avg Cy5 intensity/cell	868.7 ± 382.0	6.8 ± 17.0

Figure S13: Comparison of Retention of CPP-Anchor-Cy5 with and without washing step
The top two figures are of the CPP-anchor-cy5 in cells with no wash step. The bottom two images show the relative retention after the addition of a one hour wash step to reduce the amount of unbound CPP retained by the cells. Using ImageJ, the mean intensity of Cy5 was calculated on a per cell basis, and those numbers are given in the table below the image. Difference between the per cell fluorescence in E17K and wildtype transfected cells were not significant without washing, and significant ($p = 0.00018$) with washing. While the wash step likely will not remove all of the excess or unbound peptide, its inclusion allows for a difference in intracellular sequestration to be detected between the cell types.

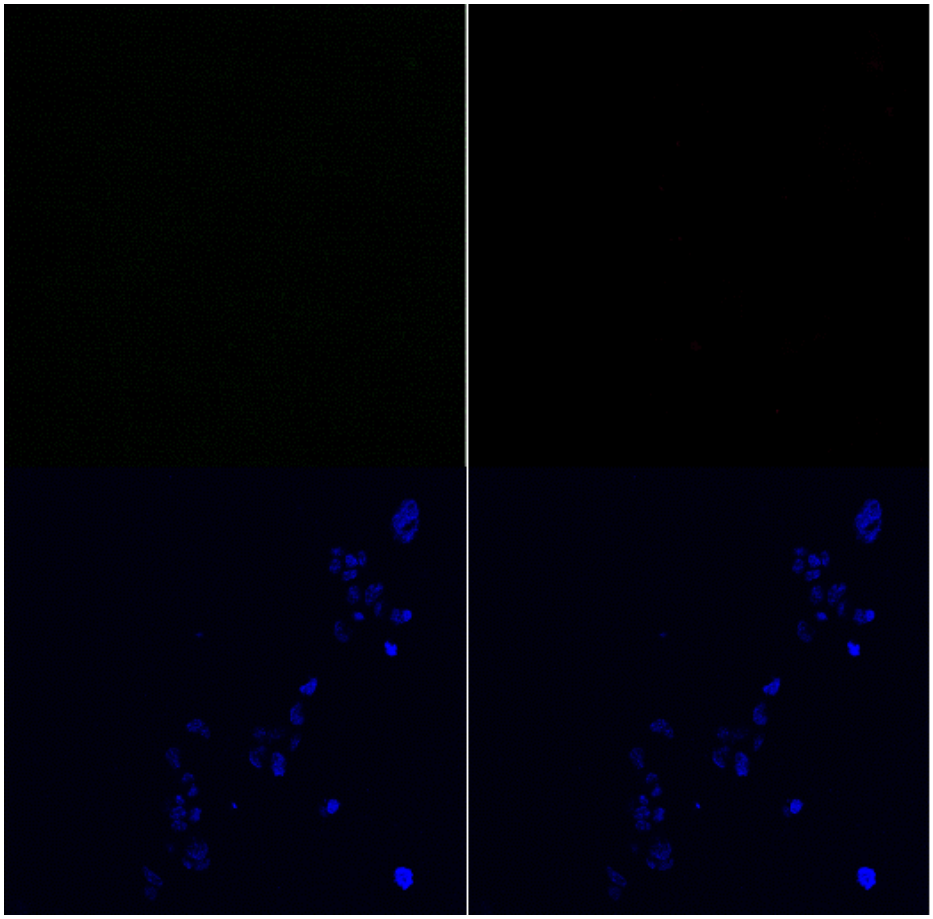


Figure S14: Control Experiment: In cell Imaging of HEK293T cells with no GFP-PH Domain expressed

This control was designed to prove that the ligand binding is due to the presence of the expressed E17K GFP-PH Domain in the cells. These cells were not transfected to express the GFP PH Domain protein and were still treated with 50nM concentration of yleaf – PEG_5 – TAT – Cy5. As in the non-control assays, live cells were treated with the labeled PCC Agent, fixed, washed, and then identically imaged. Following those protocols, neither GFP fluorescence signal nor Cy5 fluorescence was observed within the cells. If the ligand were sequestered in cells for reasons other than binding to the GFP-PH Domain, we would expect to see Cy5 signal in these cells.

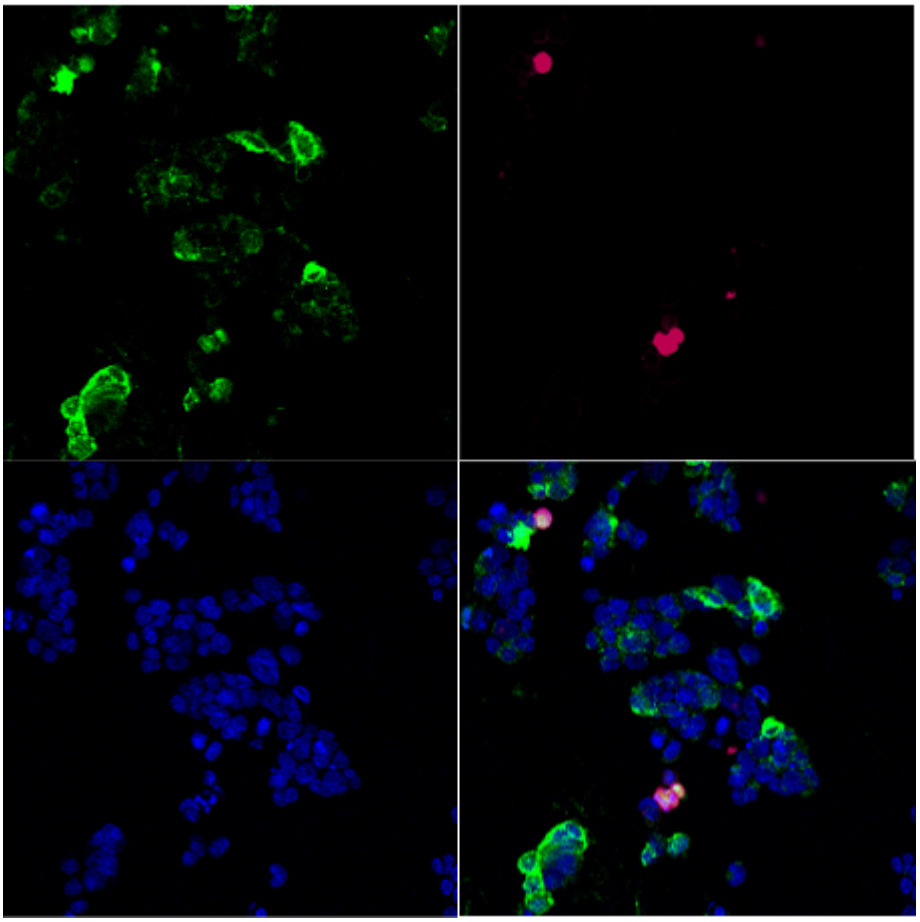


Figure S15: Control Experiment: In cell imaging PEG_5 – TAT – Cy5 control.

Cells expressing GFP- E17K mutant protein were incubated with 50nM PEG_5 – TAT – Cy5 (the yleaf ligand is missing). This control exposes the cells to all of the components of the imaging agent except for the anchor ligand peptide. As in the non-control assays, live cells were treated with the fluorescent reagent, fixed, washed, and then identically imaged. Very little Cy5 fluorescence signal is observed, consistent with the observation that the fluorescent signal in the non-control assays arises from the GFP-E17K mutant PH Domain interacting with the yleaf containing PCC Agent.

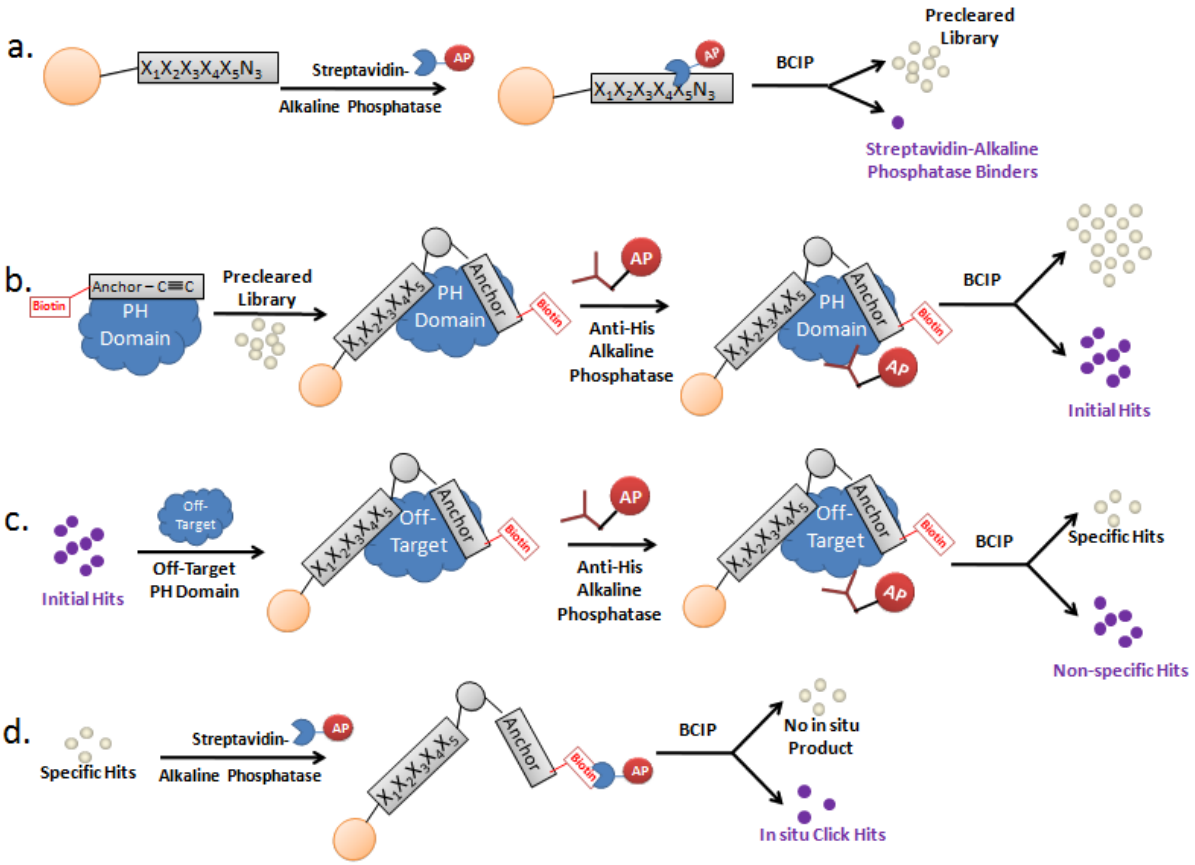


Figure S16: Screening Strategy for Biligand Determination

(a) Preclear: Library beads are incubated with streptavidin - alkaline phosphatase conjugate to remove any library beads that bind to this or the BCIP reagents. **(b)** Target Screen: Precleared beads are incubated with the target and anchor ligand and allowed to "click" to form a triazole. The presence of the target his-tagged PH Domain is detected via an anti-His alkaline phosphatase antibody. The hit beads are then collected, decolorized, and stripped of protein. **(c)** Anti-Screen: Hit beads from the target screen are incubated with the off-target PH Domain and anti-his alkaline phosphatase. These hit beads bind to both the target and off-target (WT and E17K mutant). **(d)** Product Screen: The remaining beads are probed with streptavidin-alkaline phosphatase to determine which contain the click product and, thereby, have shown biligand formation.

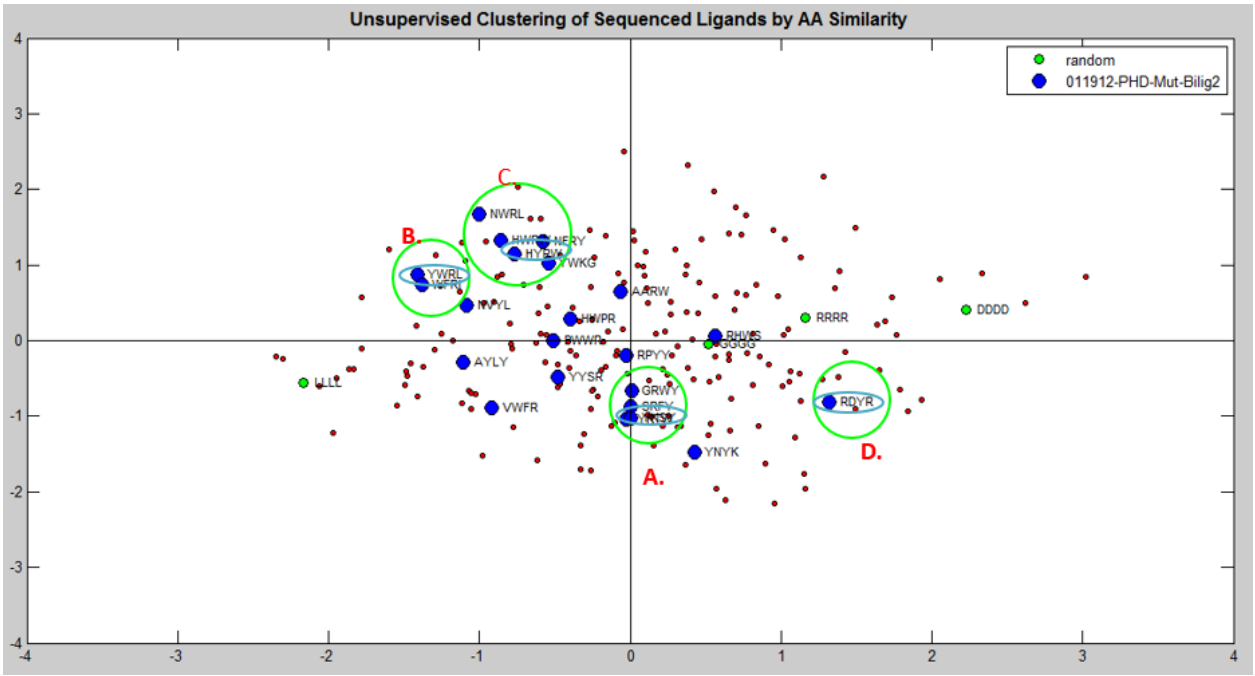


Figure S17: Unsupervised Clustering of Biligand Sequence Ligands by AA Similarity

Hit sequences from the biligand screen were analyzed by their hydrophobicity and sequence homology using principal component analysis. Clusters circled in green indicate clustered regions and the cyan circles indicate the peptide that was selected and scaled-up as a possible biligand sequence. The potential biligand sequences that were tested are: yleaf-ywrl, yleaf-yksy, yleaf-rdyr, and yleaf-hyrw, where “yleaf” is the anchor ligand and the “-“ indicates the location of the triazole linkage.

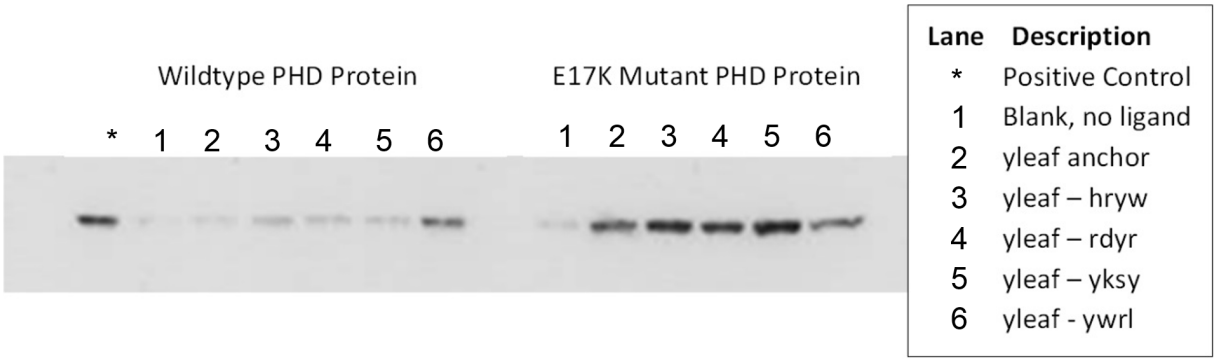


Figure S18: Immunoprecipitaiton Assay Results for Biligand Candidates

Lane 1: protein positive blot control; lane 2: WT GST-PHD, no ligand; lane 3: WT GST-PHD, yleaf anchor; lane 4: WT GST-PHD yleaf-hryw biligand; lane 5: WT GST-PHD, yleaf-rdyr biligand; lane 6: WT GST-PHD, yleaf-yksy biligand; lane 7: WT GST-PHD, yleaf-ywrl biligand. Lanes 8-13 are the same as 2-7, but with the E17K GST-PHD protein. Note that all of the biligand candidates improve upon the binding of the anchor ligand, but yleaf-yksy shows the greatest signal in binding the E17K protein and the lowest in binding the WT protein. This biligand was chosen as the candidate biligand and carried on to triligand screening.

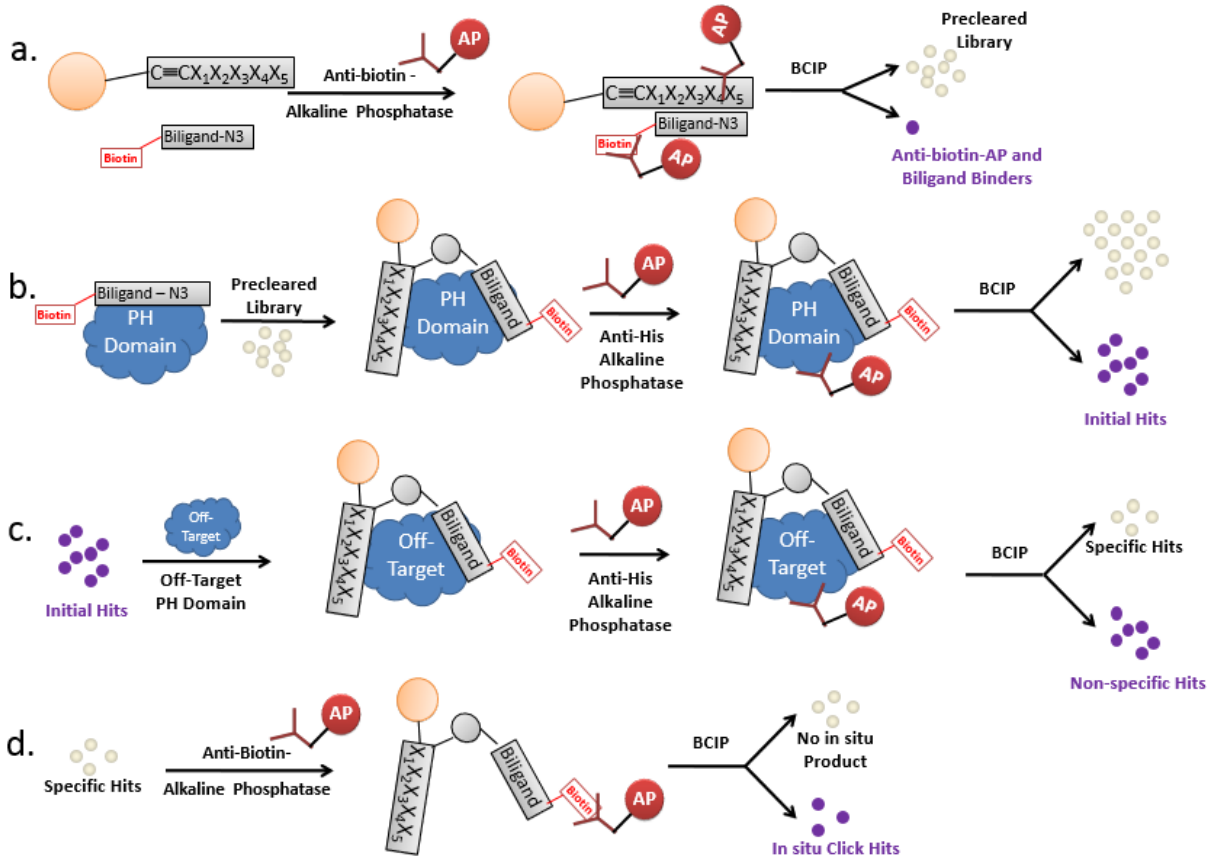


Figure S19: Screening Strategy for Triligand Determination

(a) Preclear: Library beads are incubated with streptavidin - alkaline phosphatase conjugate to remove any library beads that bind to this or the BCIP reagents. **(b)** Target Screen: Precleared beads are incubated with the target and biligand and allowed to "click" to form a triazole. The presence of the target his-tagged PH Domain is detected via an anti-His alkaline phosphatase antibody. The hit beads are then collected, decolorized, and stripped of protein. **(c)** Anti-Screen: Hit beads from the target screen are incubated with the off-target, WT PH Domain and anti-his alkaline phosphatase. These hit beads bind to both the target and off-target (WT and E17K mutant). **(d)** Product Screen: The remaining beads are probed with streptavidin-alkaline phosphatase to determine which contain the click product and, thereby, have shown triligand formation.

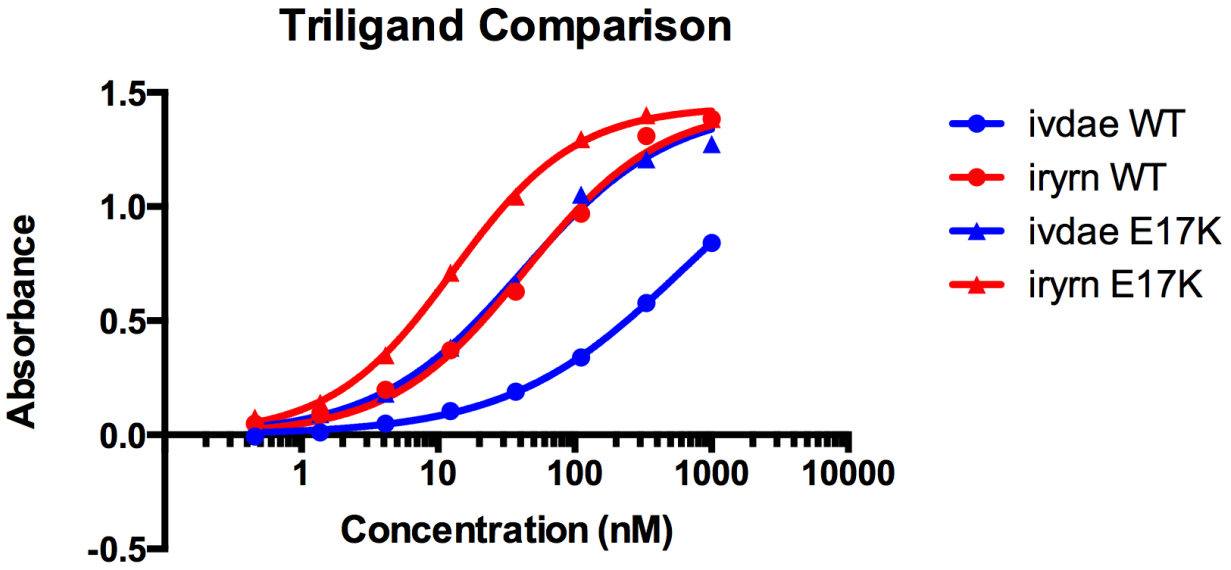


Figure S20: ELISA assay of two triligand candidates

This sandwich ELISA was performed with biotinylated triligands immobilized on a neutravidin plate. The protein was incubated in varying concentrations and detected with an anti-GST antibody. The iryrn triligand candidate, while demonstrating a higher affinity for the E17K protein than the ivdae candidate, was not chosen due to an increased binding to the off-target, WT protein. The triligand ivdae was carried forward as the final triligand PCC agent.

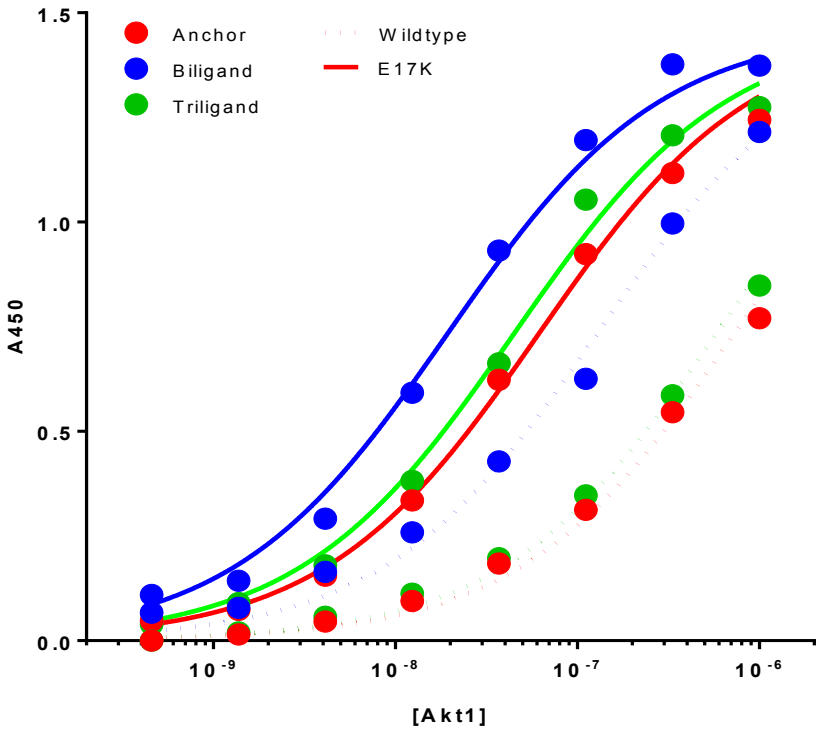


Figure S21: Full Sandwich ELISA curves of anchor, biligand and triligand against WT and E17K PH Domain

ELISA assays were performed by immobilizing biotinylated ligand onto a neutravidin coated ELISA plate. The plates were blocked with BSA, incubated with varying concentrations of GST-tagged protein and detected with an anti-GST HRP antibody.

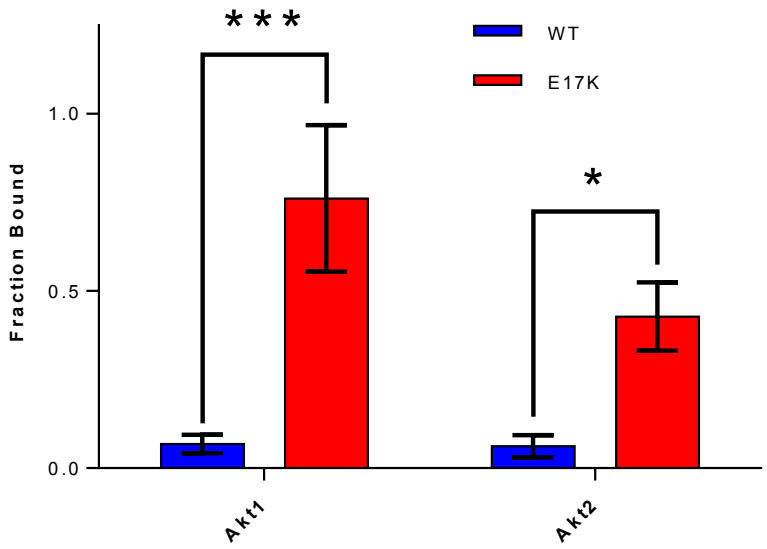


Figure S22: Point ELISA demonstrating binding to both Akt1^{E17K} and Akt2^{E17K} isoforms

ELISA assay was done as per the full curves, except only points were done at 100nM concentration of protein. The isoforms are 79% homologous as calculated by a pairwise sequence analysis using Blast2Seq between the Akt1 E17K structure (2UZR) and the Akt2 PHD structure (1P6S).

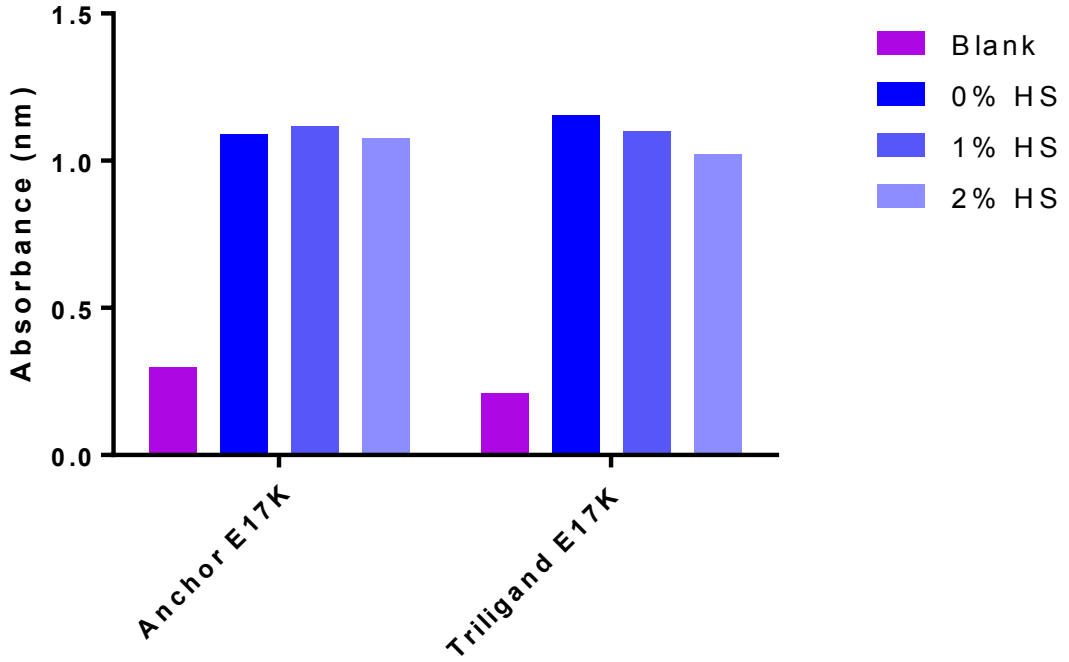


Figure S23: Performance of Anchor and Triligand in 1% and 2% serum

The anchor and triligand were tested in a point ELISA against 150nM of WT or E17K GST-Akt1 protein spiked into 0%, 1% or 2% human serum (HS) in TBS buffer. This assay uses the same format as the full-curve ELISAs in **Supplemental** *Figure S21*, and the ligand binding and specificity are consistent with what was seen in that figure.

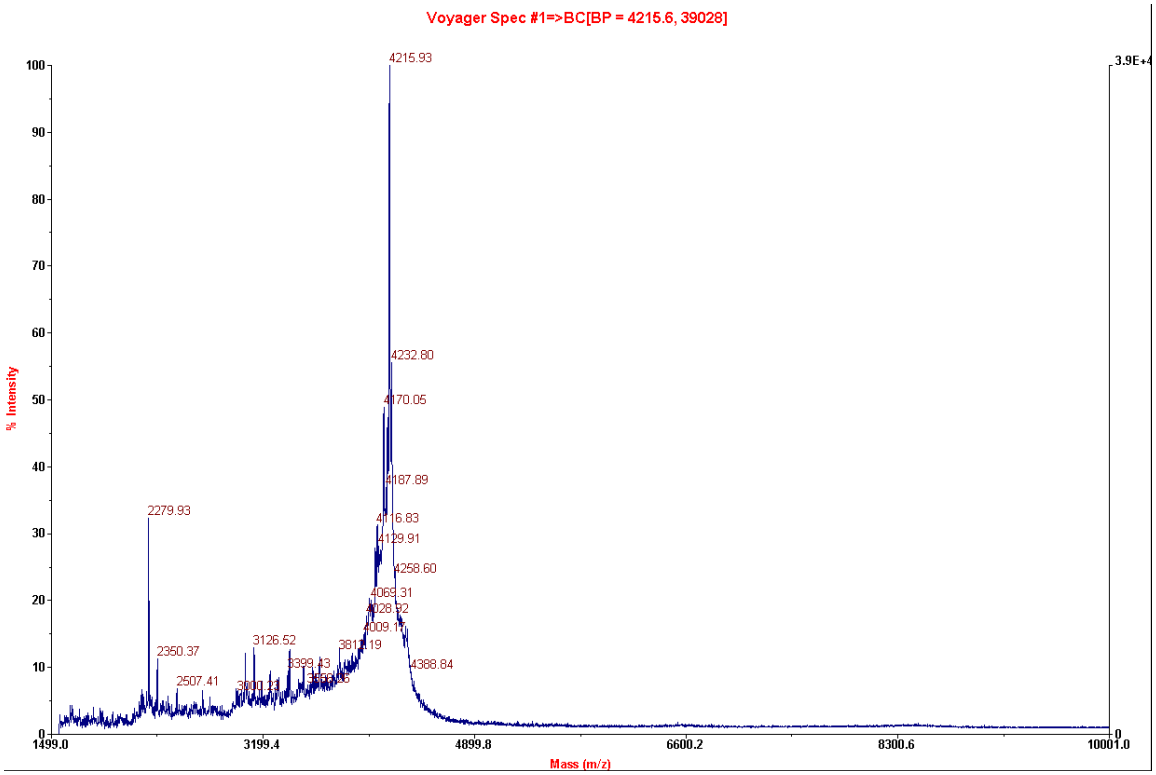


Figure S24: MALDI-TOF of 33-mer target fragment.

Sequence: Biotin- MSDVAIVKEGWLKKRGKY[Pra]KTWRPRYFLLKNDG. Expected m/z: 4214.9, observed M+H: 4215.93

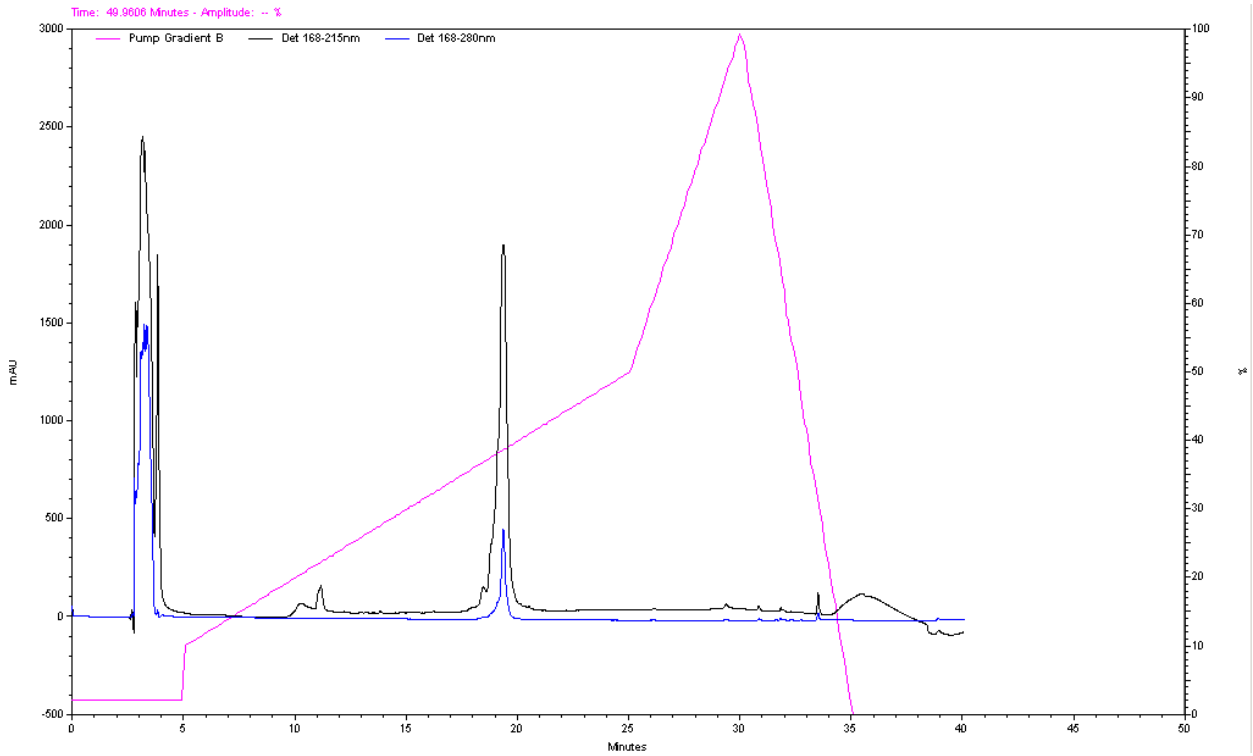


Figure S25: HPLC trace demonstrating purity of 33-mer E17K Fragment

The 33-mer fragment dissolved in buffer was run on an analytical HPLC to demonstrate the purity of the peptide that was analyzed by MALDI in Figure S1. The peak below 5 minutes is the salt from the buffer.

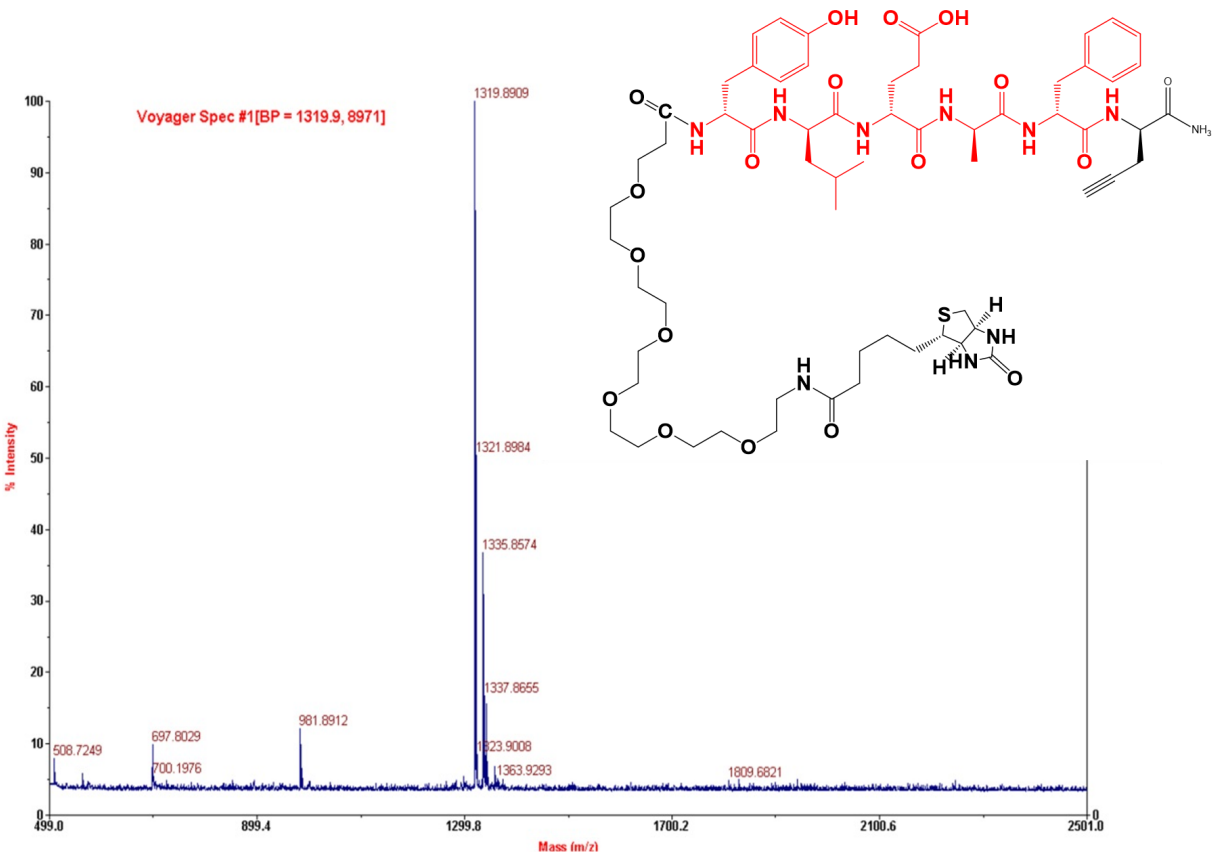


Figure S26: Structure and MALDI-TOF of Biotin – PEG_6 – yleaf – Pra anchor ligand

The anchor ligand is appended with a PEG6-biotin and a C-terminal propargylglycine alkyne amino acid for the click reaction during the screen. Expected M+Na: 1319.62, observed: 1319.89.

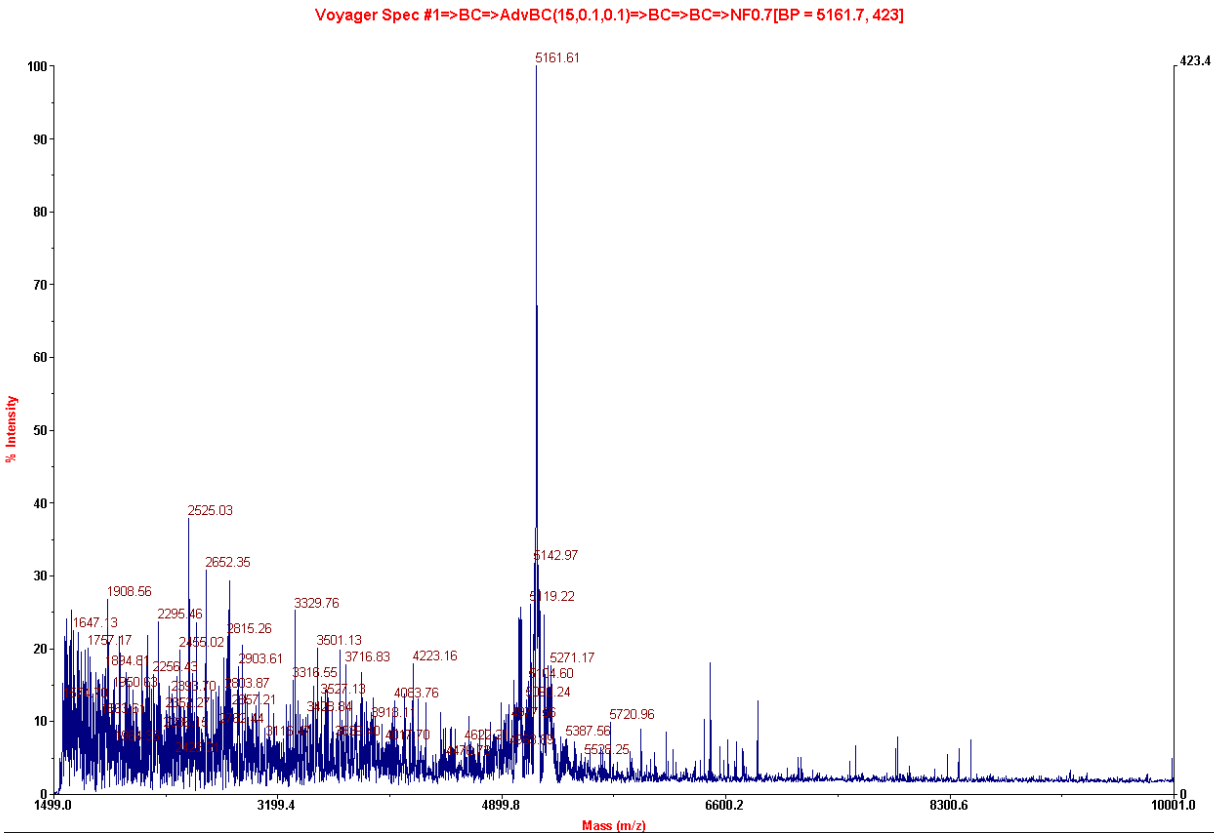


Figure S27: MALDI-TOF of 6His – PEG5 – Mut 33mer Fragment

Sequence: HHHHHH – PEG_5- MSDVAIVKEGWLKKRGKY[Pra]KTWRPRYFLLKNDG. Expected: 5160.72, observed: 5161.61.

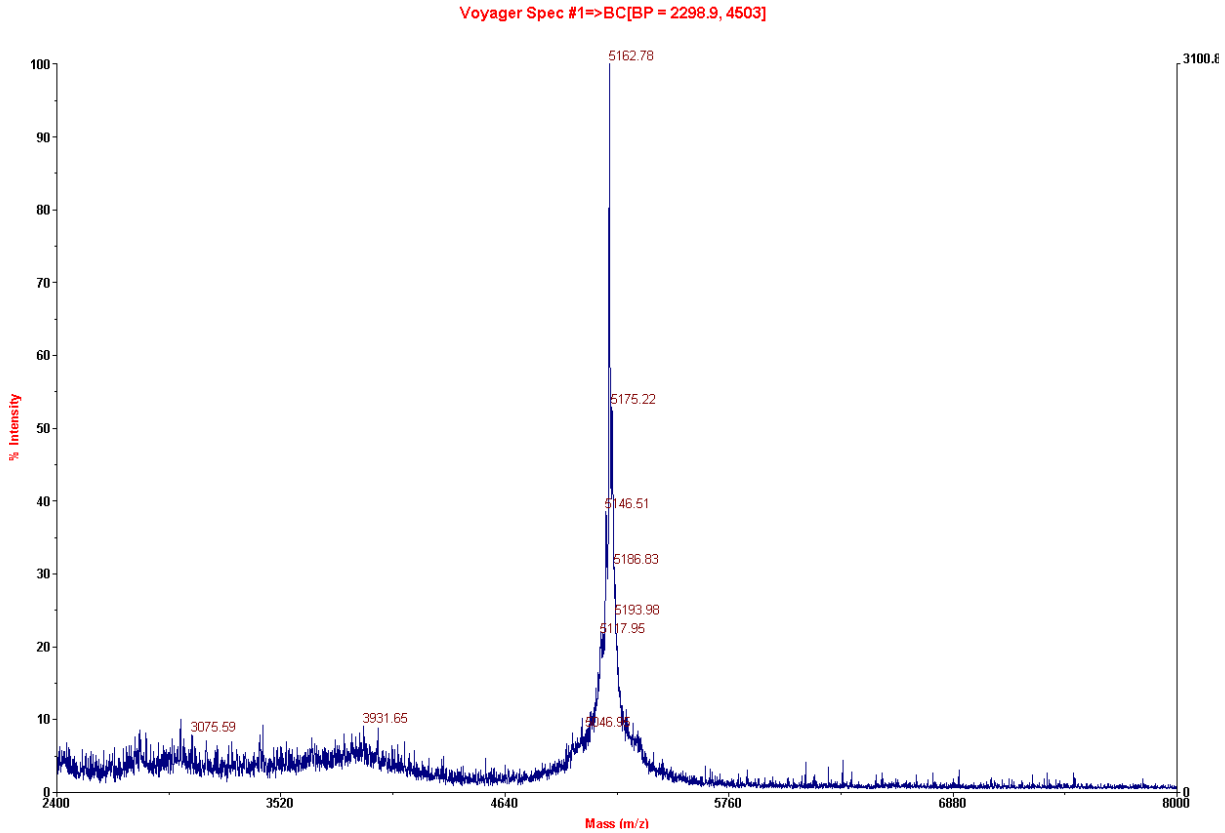


Figure S28: MALDI-TOF of 6His – PEG5 – WT 33mer Fragment

Sequence: HHHHHH – PEG_5- MSDVAIVKEGWLKKRGKY[Pra]KTWRPRYFLLKNDG. Expected: 5161.72, observed: 5162.78.

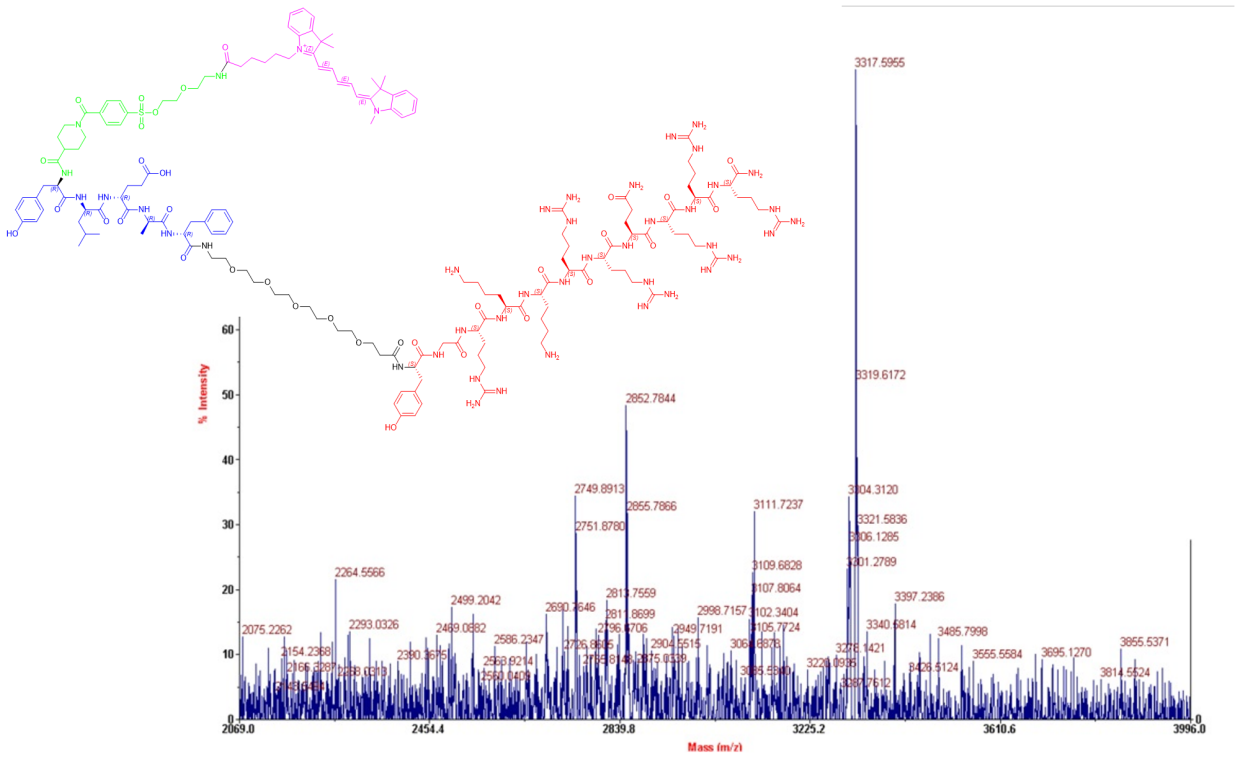


Figure S29: MALDI-TOF of CPP – yleaf – piperidine – tosyl – EG – Cy5 for labeling studies

The anchor is built onto a TAT peptide, then appended with the piperidine linker and tosyl labeling arm with a Cy5, as described above. Note, the Cy5 anchor flies very poorly on the MALDI and so background and slight impurities appear amplified. Expected: 3319, observed: 3319.6.

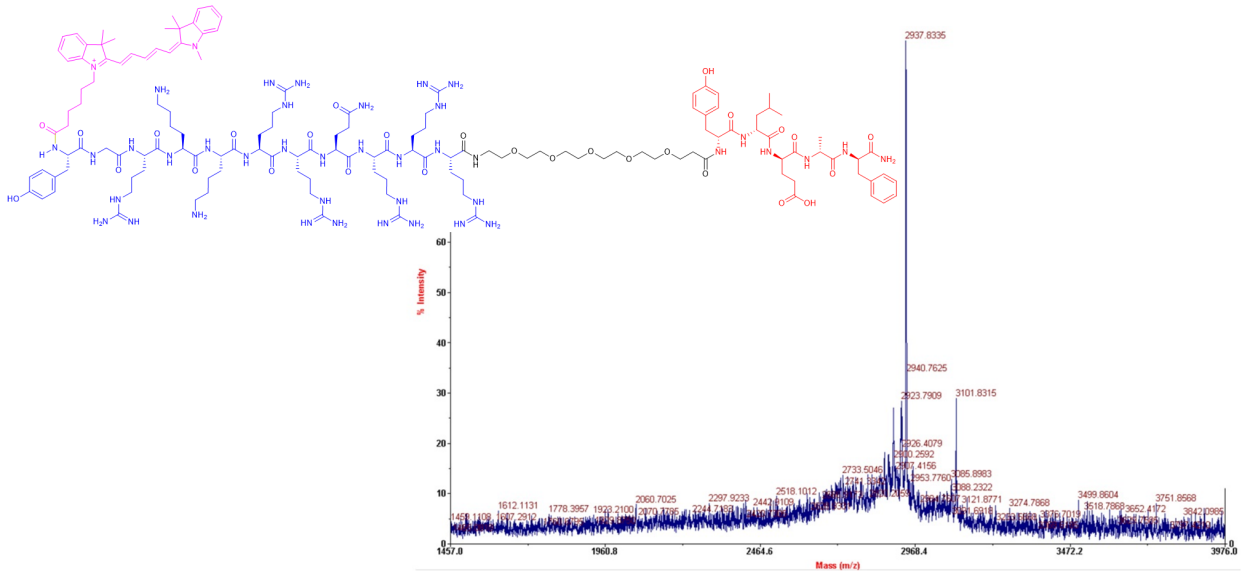


Figure S30: Structure and MALDI-TOF of yleaf – PEG_5 – TAT – Cy5 for imaging studies

The anchor ligand is appended with a PEG5-TAT, to ensure cell penetration, then a Cy5 dye is added for visualization. Expected: 2937.72, observed: 2937.83.

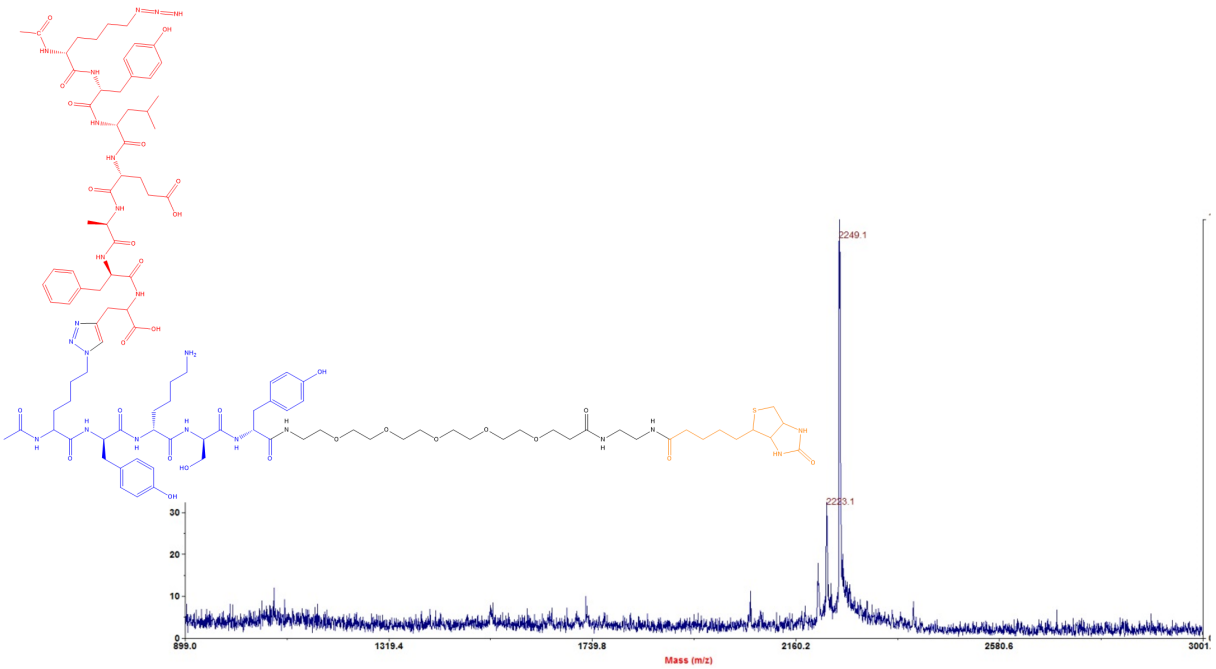


Figure S31: MALDI of Lys(N_3) – yleaf – yksy – PEG_5 – Biotin Biligand

The biligand is appended with a C-terminal PEG_5-Biotin for detection in the screen and an N-terminal Lys(N_3) for use in the *in situ* click screen. Expected: 2248.1, observed: 2249.1.

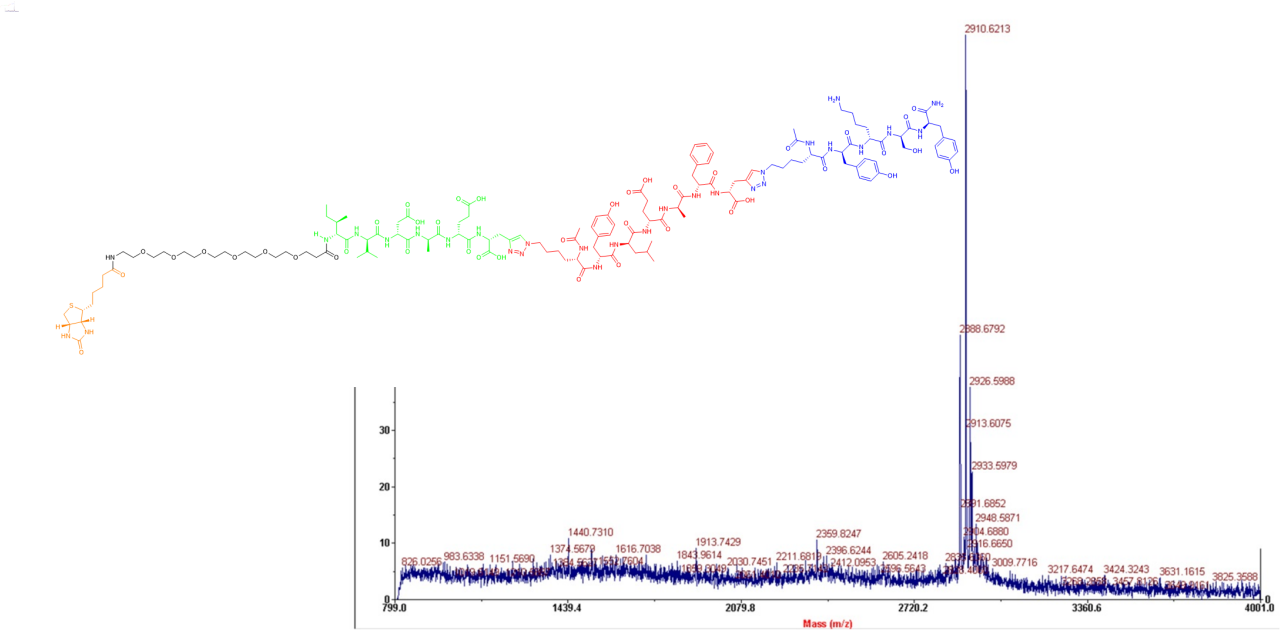


Figure S32: Structure and MALDI-TOF of Biotin – PEG_5 – ivdae – yleaf – yksy Triligand

The triligand was appended with a biotin tag for detection. Expected: 2888.39, Observed: 2888.68 (M+H), 2910.62 (M+Na).

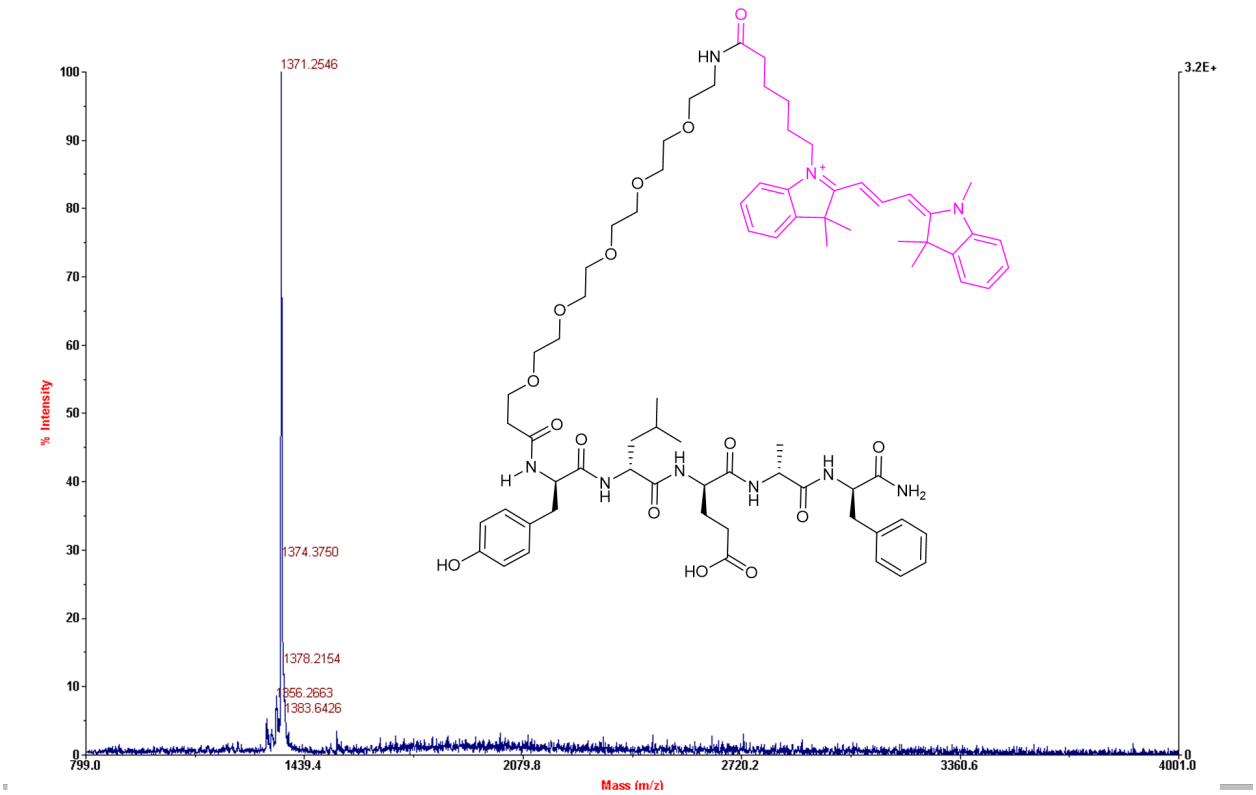


Figure S33: Structure and MALDI-TOF of Cy3-PEG$_5$-yleaf for fluorescence polarization

The yleaf anchor was appended with an N-terminal PEG$_5$ and a Cy3 dye. Expected: 1371.75, Observed: 1371.25.

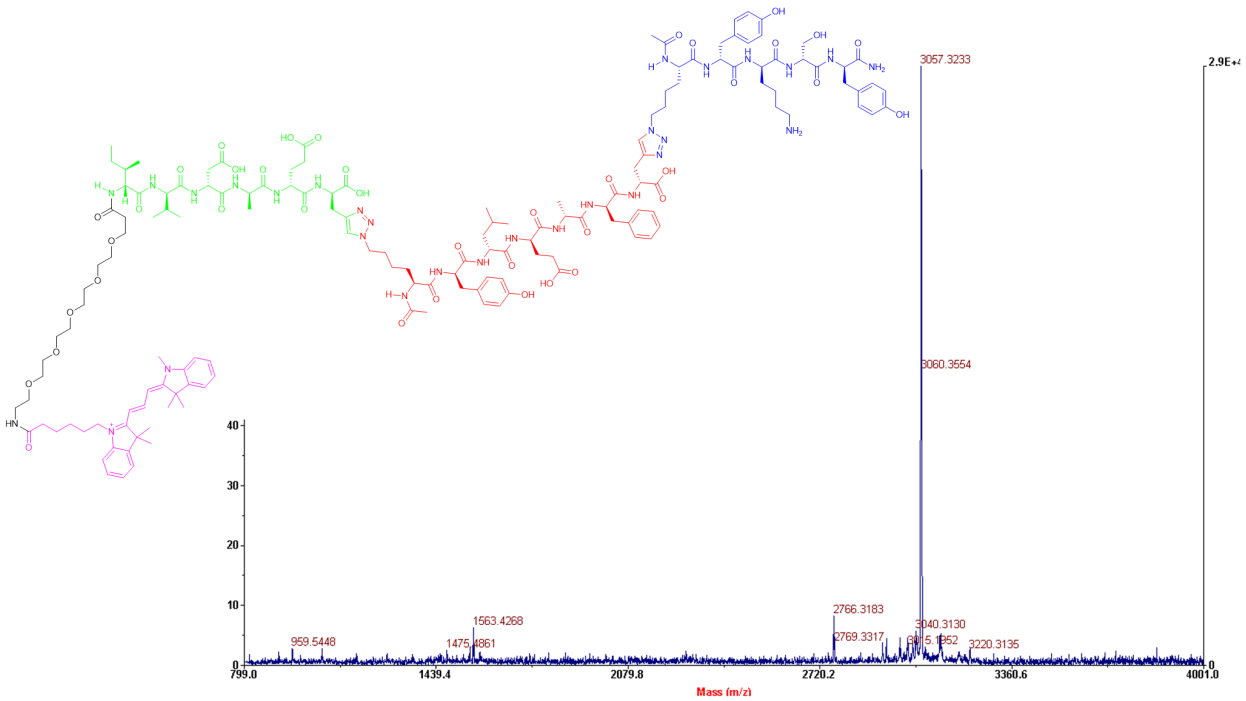


Figure S34: Structure and MALDI-TOF of Cy3-PEG5-ivdae-yleaf-yksy triligand for fluorescence polarization

The triligand was synthesized as usual and was appended with an N-terminal PEG5 and Cy3. Expected: 3057.56, observed: 3057.32.

Table S1: Hit sequences from Anchor Screen Against 33-mer Epitope (5hr)

Note that the missing or unclear sequences are due to an error in the ProciseCLC Edman degredation machine and not the library itself. Therefore, they are counted in the statistic, but are not used due to the uncertainty in the sequence.

Az2	G	v	e	k	f
Az8	y	h	e	w	f
Az4	i	s	e	y	e
Az2	p	h	w	l/k	f
Az8	d	l	l	t	f
Az4	a	r	s	d	f
Az8	f	k/l		G	t
Az8	f	e	i	q	
Az8	e	e	p	d/n	f

Table S2: Hit Sequences from Anchor Screen Against 33-mer Fragment (overnight)

Az4	e	e	f	e	f
Az8	f	e	e	a	i
Az2	e	l	n	h	y
Az2	h	a	r	h	q
Az2	h	e	w	v	t
Az4	n	w	y	a	w
Az4	n	l	v	p	n
Az2		r	r	r	f
Az4	a	l	n	s	k
Az8	p		a	y	h
Az2	n	r	y	v	r
Az8	y	l	e	a	f

Table S3: Tryptic fragment data workup for labeling experiment

MALDI Peak	Peak - dye		Expected	Digest	Peak Area	P/M 1		Corresponding Fragment
1053.15	*500.78*		*1051.6349*	*499.265*	*4296.69*	*1.5151*		
1090.15	537.78		*1114.6007*	562.2307	5813.86	24.4507		
1118.11	565.74		*1114.6007*	562.2307	12649.91	3.5093		
1142.16	*589.79*		*1132.6993*	*580.329*	*4217.63*	*9.4607*		
1179.14	*626.77*		*1173.6565*	*621.287*	*4393.2*	*5.4835*		
1194.14	641.77		*1201.732*	649.362	5139.51	7.592		
1202.16	*649.79*		*1201.732*	*649.362*	*4103.69*	*0.428*		
1234.66	682.29	*	*1234.7826*	682.4126	8193.47	0.1226		YFLLK
1300.08	747.71		*1303.7273*	751.3573	6445.8	3.6473		
1302.09	*749.72*		*1303.7273*	*751.357*	*4496.81*	*1.6373*		
1308.09	755.72		*1303.7525*	751.3825	5926.62	4.3375		
1320.57	768.2	*	*1320.7691*	768.3991	7886.31	0.1991		EGWLHK
1440.11	887.74		*1447.8246*	895.4546	6406.74	7.7146		
1475.16	922.79		*1477.9158*	925.5458	10131.17	2.7558		
1493.13	940.76		*1477.9158*	925.5458	9276.21	15.2142		
1499.13	*946.76*		*1507.814*	*955.444*	*4112.05*	*8.684*		
1515.1	962.73		*1507.814*	955.444	4687.71	7.286		
1567.65	1015.28		*1565.8591*	1013.489	7907.73	1.7909		
1639.2	1086.83		*1645.9403*	1093.57	21961.13	6.7403		
1707.53	1155.16		*1701.0101*	1148.64	12923.9	6.5199		
1791.09	1238.72		*1795.9606*	1243.591	5200.25	4.8706		
1802.79	1250.42		*1800.0105*	1247.641	8149.76	2.7795		
1851.79	*1299.42*		*1841.9813*	*1289.61*	*4331.77*	*9.8087*		
1995.47	*1443.1*		*1957.1459*	*1404.78*	*4368.22*	*38.3241*		
2212.04	1659.67	*	*2213.208*	1660.838	95735.94	1.168		EEWTTAIQTVADGLK
2225.51	1673.14		*2213.208*	1660.838	17712.89	12.302		
2233.95	1681.58		*2213.208*	1660.838	12256.12	20.742		
2284.12	1731.75		*2344.242*	1791.872	5711.7	60.122		
2306.92	1754.55		*2344.242*	1791.872	6553.24	37.322		
2344.23	1791.86	*	*2344.242*	1791.872	4506.1	0.012		EAPLNNFSVAQCQLMK
2383.46	1831.09		*2362.2571*	1809.887	8608.79	21.2029		

Table S4: Hit Sequences from Biligand Screen

Az4	h	w	p	r
Az4	n	v	y	l
Az4	h	y	r	w
Az4	r	d	y	r
Az4	y	n	y	k
Az4	y	k	t	w
Az4	s	r	f	y
Az4	y	k	s	y
Az4	y	y	s	r
Az4	r	h	w	s
Az4	p	w	w	r
Az4	n	f	r	y
Az4	y	w	r	l
Az4	y	w	k	G
Az4	a	y	l	y
Az4	h	w	r	w
Az4	n	w	r	l
Az4	a	a	r	w
Az4	G	r	w	y
Az4	w	f	r	i
Az4	r	p	y	y
Az4	v	w	f	r

Table S5: Hit Sequences from Triligand Screen

G	l	-	-	m	-
i	r	y	r	n	Pra
i	v	d	a	e	Pra

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1. Coin, I.; Beyermann, M.; Bienert, M., Solid-phase peptide synthesis: from standard procedures to the synthesis of difficult sequences. *Nature protocols* **2007,** *2* (12), 3247-56.

2. Corrêa, D. H.; Ramos, C. H., The use of circular dichroism spectroscopy to study protein folding, form and function. *African J Biochem Res* **2009,** *3* (5), 164-173.

3. Lee, S. S.; Lim, J.; Tan, S.; Cha, J.; Yeo, S. Y.; Agnew, H. D.; Heath, J. R., Accurate MALDI-TOF/TOF sequencing of one-bead-one-compound peptide libraries with application to the identification of multiligand protein affinity agents using in situ click chemistry screening. *Anal Chem* **2010,** *82* (2), 672-9.

4. Tsukiji, S.; Miyagawa, M.; Takaoka, Y.; Tamura, T.; Hamachi, I., Ligand-directed tosyl chemistry for protein labeling in vivo. *Nat Chem Biol* **2009,** *5* (5), 341-343.